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Morphological and serological characterization of bacterial isolates from greening/dieback diseased citrus

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MORPHOLOGICAL AND SEROLOGICAL CHARACTERIZATION OF BACTERIAL ISOLATES FROM GREENING/DIEBACK DISEASED CITRUS

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ABSTRACT

The morphological and serological characteristics of bacterial isolates from plants infected with African greening, Reunion greening and Taiwan likubin were investigated. Isolates from Australian citrus dieback affected trees and resembling the putative greening isolates, were also included. Although predominantly long thin rods, the bacterial cells were morphologically variabile in both liquid and plate cultures at 25°C and 35°C. "Round forms" developed with age and nutrient limitations. Based on colony morphology and pigmentation, the isolates were categorized into two groups - Groups 1 and 2. Whole cell protein patterns were obtained by SDS-PAGE. Patterns of the Group 1 isolates, which were conserved with growth, were similar to one another and different from the patterns of the Group 2 isolates. In an attempt to establish a bacterial detection probe, antisera were raised against the Group 1 isolates. These sera specifically reacted with all the members of this group in slot-blot immunoassays. Using the sera in western blots, characteristic serological reaction patterns were associated with the Group 1 and Group 2 isolates. Both greening-affected and dieback-affected field samples reacted specifically with the antisera in slot-blot immunoassays. A monospecific polyclonal antiserum was also raised against a 38K - 40K protein band in western blots of the Group 1 isolates. The reaction of antigenic bands in western blots of preparations of tissue from affected trees can be achieved although the techniques involved need to be refined. Metabolically, the Group 1 isolates are somewhat related to Clavibacter michiganense subsp. michiganense. However, the protein pattern and western blot results did not support this. A Clavibacter sp. was isolated from Australian citrus dieback affected trees. A Group 1 isolate was also obtained from Australian citrus dieback affected trees.

Declaration

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfilment of the requirements for the degree of Doctor of Philosophy. The material presented is the result of my own unaided work and has not previously been submitted at another university or institution.

Daniel C. Böck

9 March, 1991

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TE SALUDO, UNA AVENTURA NUEVA

CON TODA MI ANSIA,

POR SATISFACER MI DESEO MAS PROFUNDO...

VIVIR Y ESTAR CONTENTO!

PUEDA SER PROSPERO,

Y ASI REALIZAR MI FELICIDAD.

PUEDA SER DIFICIL,

PARA QUE MI LUCHA POR EL DESAFIO PUEDA

SURGIR A LA SUPERFICE.

TAL ES LA VIDA,

PARA VIVIR, ESTAR CONTENTO Y SATISFECHO;

PARA QUE MAÑANA PUEDA DECIR QUE HOY

HE PODIDO REALIZARME.

Laura D. M. Böck

1988

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LIST OF ABBREVIATIONS

A.A.H.L	- Australian Animal Health Laboratories
ACD	- Australian citrus diedack
ANOVA	- Analysis of Variance
APS	- Ammonium persulphate
BCIP	- 5-bromo-4-chloro-3-indolyl-phosphate
BCRI	- Biological and Chemical Research Institute, N.S.W., Australia
CTV	- Citrus tristeza virus
d.f.	- degrees of freedom
GAR-AP	- Goat anti Rabbit - Alkaline phosphatase conjugate
IM	- Intra muscular
IV	- Intra venous
LMW	- Low molecular weight
LPS	- Lipopolysaccharide
MA	- Murray river area
MIA	- Murrumbidgee river irrigation area
MIG	- Medium for the isolation of Greening
MS	- Mean of squares
NA	- Nutrient agar
NBT	- Nitro blue tetrazolium
NGA	- Nutrient glucose agar
PAGE	- Polyacrylamide gel electrophoresis
PBS	- Phosphate buffered saline
SDS	- Sodium dodecyl sulphate
SPA	- Sucrose peptone agar
SS	- Sum of squares
TBS	- Tris buffered saline
TEMED	- N,N,N'N'Tetramethylethylenediamine

TTBS - Tween tris buffered saline

UNSW - University of New South Wales

U.S.D.A. - United States Department of Agriculture

v/v - Volume/volume

WU - Wollongong University

w/v - Weight/volume

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1.0 INTRODUCTION

1.1 Greening – What is it ?

Greening is one of the most serious diseases of citrus occurring in many of the citrus growing regions of Africa and South East Asia. Because of its geographical distribution and the failure to recognise the similarity of the disease in the many affected areas, it is known by a variety of names including citrus yellow shoot, likubin, dieback, leaf-mottle or leaf-mottle-yellows, vein phloem degeneration, yellow branch and blotchy mottle. The term "greening", originally derived from the greened appearance of the fruit, is now, however, readily accepted, although the term "blotchy mottle" might have been more appropriate (McClean and Oberholzer, 1965).

The many aspects of citrus greening worldwide have been extensively reviewed (da Graça, in press).

An overview is provided of the literature that led to this research, the results of which it is hoped, will contribute to the overall understanding of citrus greening disease.

1.2 Symptomology

In the early stages of infection, greening symptoms are usually limited to single branches which are conspicuous because of the chlorotic appearance of the leaves (McOnie, 1982; Fraser et al., 1966). In fact, greening can be highly sectorial. A tree may have a single affected branch or, conversely, a totally affected tree may have a single healthy branch for the duration of its life (Moll and van Vuuren, 1982). Severely affected trees may be totally unproductive, appearing stunted,

with a sparsity of foliage and bearing abnormal fruit of poor quality (McOnie, 1982; McClean and Oberholzer, 1965).

Establishment of the disease is first noticed by the the sectorial discolouration of the leaves of some branches. Secondary growth from infected branches results in the development of both weak and strong shoots. Stronger shoots may be asymptomatic and falsely suggest a recovery from the disease (McClean and Oberholzer, 1965). Both the strong and weak shoots, however, remain affected. Leaves fall off prematurely, multiple buds develop on the defoliated branches and excessive blossoming can occur (McOnie, 1982). A dieback of the branches follows (McOnie, 1982; McClean and Oberholzer, 1965).

The fibrous root systems of affected trees, although not visibly affected, are less extensive; the quantity of roots are usually insufficient to support the tree (McClean and Oberholzer, 1965).

1.2.1 Leaves

Young developing leaves of a new flush may appear slightly paler than usual in affected trees. Symptoms become more pronounced as the leaves mature and are readily observed on the older leaves back in the tree (Fraser et al., 1966; McClean and Oberholzer, 1965; McClean and Schwarz, 1970). Most distinctive of the leaf symptoms is the yellowing of the midrib and larger lateral veins. The subsequent spread of this discolouration into the adjacent interveinal tissue produces a mottling effect (Fraser et al., 1966; McClean and Oberholzer, 1965).

The leaves associated with secondary growth are small, narrow and upright, with discolourations that resemble nutrient deficient conditions (Fraser et al., 1966; McClean and Schwarz, 1970). These leaves become leathery in texture and develop

prominent veins. The extent of the discolouration varies. The leaves may be pale green with a distinctly darker green venation; have irregular blotches of green or green spots on an otherwise yellow lamina and/or be completely chlorotic (Fraser et al., 1966; McClean and Schwarz, 1970). Premature abscision of these leaves usually occurs.

1.2.2 Fruit

The fruit remain small and lopsided and have been described as acorn shaped. The side away from the sun remains green and develops more slowly (McOnie, 1982; McClean and Oberholzer, 1965). Most of the fruit will drop off severly affected trees at any time during development. Those remaining on the tree rarely attain full size and are unevenly coloured.

Internally, the fruit are a dirty brown. They are of poor quality and salty bitter to the taste. The seeds are usually aborted although larger fruit may contain some seed most of which are sterile, small and brown in colour (McOnie, 1982; McClean and Oberholzer, 1965). This brown colouration is attributed to the partial development of the outer seed coat which fails to surround the inner brown coat (Schneider, 1968).

1.2.3 Anatomical

The internal effects of infection occur in the vascular system of maturing and older leaves. Localized areas of necrotic phloem and associated hypertrophy as well as hyperplasia of the adjacent parenchyma cells occur in the petiole, midrid, lateral veins, and veinlets, affecting leaf anatomy and colouration (Schneider, 1968).

Secondary changes include the enlargement of the starch granules in the chloroplasts of the palisade parenchyma cells to the point where the entire cell is filled and both the grana and chlorophyll are destroyed. This results in the leathery/corky texture of the leaf and angular blotching of the lamina. Cambial activity within the veins becomes disorderly and excessive replacement phloem is formed to bypass the blockages. These new phloem cells may vary in shape from normal to highly irregular and eventually also become necrotic (Schneider, 1968).

New shoots that arise from the axillary buds of affected leaves do not develop properly. The disturbed state of the older shoot from which they originate interferes with the nutrient translocation to these new shoots. The fruit on affected branches similarly do not receive the carbohydrates that are produced in the leaves and that are required for normal development (McClean and Schwarz, 1970).

1.3 Aetiology

Based on the symptomology, citrus greening disease was first believed to be a nutrient deficiency condition caused mainly by insufficient zinc (Zn), although a limited availability of manganese (Mn) and iron (Fe) were considered contributing factors. Zn deficiency is characterized by bright, creamy/white to yellow blotches developing on the leaves that contrast with the green colouration along the veins. The leaves remain small, narrow, pointed and erect. Severe deficiency leads to the development of short shoots with clusters or rosettes of small leaves. The whole tree appears bushy and upright with a heavy dieback of twigs. Zn deficiency is also known as "little leaf", "rosette" or "mottle leaf" (Beattie et al., 1984).

Failure to relieve these symptoms by supplementation with fertilizers, and the subsequent transmission of the causal factor of the disease by grafting, suggested

that this causal agent was a virus or virus-like particle (VLP). Reference to the greening pathogen as viruliferous is still made (Pandey, 1986).

Mycoplasma-like organisms (MLO's) were observed in the phloem cells of citrus plants affected with African and Asian greening diseases (Laflèche and Bové, 1970[1+2]; Chen et al., 1971). Some of these organisms were spherical, measuring 100 - 200nm in diameter; others were filamentous measuring up to 2µm long (Laflèche and Bové, 1970[1+2]). MLO's were believed to be associated with greening and claims were made about their isolation and culture (Ghosh et al., 1971). However, similar structures were also associated with citrus stubborn disease (Garnier et al., 1976).

The organisms associated with greening and stubborn are not alike. The greening-associated structures are more rigid, longer and filamentous compared to the rounder, pleomorphic, sometimes dumb-bell-shaped structures associated with stubborn (Garnier et al., 1976). The envelope structure of the organism associated with stubborn consists of a unit membrane (9 - 12nm wide), while that observed in the greening organism is twice as thick and bears a closer resemblance to the rickettsia-like organisms (RLO's) and Gram-negative plant pathogens (Moll and Martin, 1974). Mycoplasmas characteristically totally lack a cell wall or any cell wall precursors. They are bounded by a single trilaminar plasma membrane sometimes covered by a thin electron dense layer presumed to be extracellular or capsular material associated with pathogenicity (Razin, 1983). The organism associated with stubborn was isolated, cultured, extensively characterized and found to be a true mycoplasma identified as Spiroplasma citri (Garnier et al., 1976).

Currently strong evidence supports the prokaryote-like nature of the aetiological agent of South African and Reunion greening, India citrus decline, Philippines leaf mottling and Taiwan likubin. Based on in situ electron microscopy, bacterial-like

bodies in the phloem cells of affected plants are elongated, measuring 300nm wide and up to 3000nm long (Moll and Martin, 1974; Bové et al., 1980; Garnett, 1985). The cell wall, about 20 - 30nm wide, consists of a double track outer membrane (±8nm) separated from a double track inner membrane (±8nm) by an electron transparent zone 5 - 15nm wide (Moll and Martin, 1974). No peptidoglycan layer was visible. The RLO cell envelope is, however, much wider (30 - 40nm). Although the membrane-like outer cell wall and cytoplasmic membrane are comparable in width to that of the greening-associated organism, the electron transparent zone measures 15 - 25nm in width and contains a third 8nm wide double track membrane. Based on these ultrastructural observations, the putative greening organism was consequently referred to as a "bacterium-like organism" (Moll and Martin, 1974).

The presence of peptidoglycan was suspected since the inner layer of the outer membrane was thicker in some areas than others. The presence of a peptidoglycan layer was confirmed indirectly (Bové et al., 1980). Penicillin and tetracycline treatments of greening affected plants caused a remission in the disease symptoms. It was suggested that the peptidoglycan layer was present but too thin to be resolved under the electron microscope. Only a thin peptidoglycan layer is required for an organism to remain flexible and pleomorphic and pass through the pores of the sieve plates (Garnier et al., 1976). The term "gracilicute-like organism" was then adopted. The peptidoglycan layer was directly observed in papain treated citrus material and the envelope structure is almost identical to that of Escherichia coli while differing from that of Staphylococcus aureus (Garnier et al., 1984). The greening-associated organism is therefore a true bacterium of the Gram-negative type.

1.4 Transmission

Organisms found in the haemolymph of psyllids feeding on affected plants were identical in structure to those in the plant phloem cells. It appears that the greening-associated organism is taken up during feeding and multiplies in the haemolymph. Their presence in the salivary gland cells suggests that they could make their way into the salivary ducts and so be transmitted back into citrus during subsequent feeding (Moll and Martin, 1973; Chen et al., 1973).

The causal agent of greening is transmitted in African countries south of the Sahara by the psyllid Trioza erytreae (Del Guercio) and throughout Asia by the psyllid Diaphorina citri (Kuwayama) (Capoor et al., 1967; Schwarz et al., 1970; Aubert et al., 1984). Although geographically delimited, T. erytreae is able to transmit the causal agent of Asian greening, as is D. citri able to transmit the causal agent of African greening (Lallemand et al., 1986). Both psyllids exist in Reunion, Mauritius and Saudi Arabia. Based on the distribution of these psyllids on Reunion island, T. erytreae does not tolerate hot and dry climates and so is restricted to the cooler elevated areas (Aubert et al., 1984; Lallemand et al., 1986). D. citri, on the other hand, resists high temperatures and low humidities and so occurs in both the cool elevated areas and hot coastal zones (Aubert et al., 1984).

Psyllids are weak fliers easily blown by winds and prone to high altitude transport. Active migration against prevailing winds also occurs (Gottwald et al., 1989). These dispersal flights depend on favourable climatic conditions, the presence of natural parasites and growth flushes (Aubert et al., 1984). From an originally random distribution the psyllids cluster and move from tree to tree. Biological control of the insect vectors to prevent the spread of the greening causal agent was implemented in Reunion as both the African and Asian forms of the disease as well as the presence of both vectors prevail. Two chalcid hymenoptera, Tetrastichus

radiatus (Waterston) a parasite of D. citri and Tetrastichus dryi (Waterston) a parasite of T. erytreae, were introduced. The result has been a sharp decrease in the occurrence of the disease.

1.5 Possible strains

The terms African greening and Asian greening mentioned earlier, suggest that there are possibly two types of the disease. Climatic effects not only influence the distribution of T. erytreae and D. citri but also the distribution and severity of the greening disease. In fact temperature is an important factor in the overall occurrence of the disease and consequently presumed to have an effect on the causal agent.

Periwinkles infected with greening-associated bacteria from a South African source and grown at 25°C developed symptoms, while the periwinkle plants remained asymptomatic at 32°C (Garnier and Bové, 1983). Infected with greening-associated bacteria from Asian source material, symptoms developed at both temperatures. Expression was, however, more severe at the higher temperature. A regression in symptom development occurred when periwinkle plants affected with African greening-associated bacteria were transferred from 25°C to 32°C.

In similar experiments, symptoms on Eureka lemon in South Africa were more severe in plants maintained at 20°C, milder at 25°C and absent at 30°C (Labuschagne and Kotzé, 1988). Symptoms did not recur in plants that were kept at 30°C and then transferred to 20°C suggesting the eradication of the bacterium.

Greening diseases are referred to, therefore, as African form and Asian form. Schwarz (1972), comparing the disease in affected plants from different areas within South Africa that were maintained under identical conditions, found that

there were two strains of the putative greening pathogen in South Africa; a severe, highly systemic strain and milder latent strain that appears not to induce symptoms under any conditions.

This observation was further emphasised by Mochaba (1988). Successful isolations and subsequent growth in culture of the putative greening pathogens were temperature dependant. Some of the South African isolates grew optimally at 25°C while others grew more favourably at 35°C. These observations may suggest that both types of the disease exist in South Africa and the isolates grown at the higher temperature belong to the same group as Asian greening. In fact, results of the effects of temperature on putative Asian isolates in culture confirm this view (Mochaba, 1988).

1.6 Isolation of the causal agent

Ultrastructural observations of greening-affected citrus leaf midribs and vector haemolymphs have contributed greatly towards the characterization of the pathogen in situ (Moll and Martin, 1974). Not only was the cell shape and size elucidated, but detailed observations of the cell wall made. Cell division, sites of infection within the phloem cells and vector relationships were also investigated and compared to other plant pathogens.

However, to further adequately characterize the greening-associated bacterium, it became necessary to obtain it in pure culture. Attempts to culture the bacterium using media as for fastidious spiroplasmas (Jones et al., 1977) and xylem limited bacteria (Davis et al., 1980), were unsuccessful. Presumably the low concentration of the bacterium in citrus tissue may have been a contributing factor.

The greening-associated bacterium multiplies to high titres in dodder (Ghosh et al., 1977) and has successfully been transmitted from sweet orange to periwinkle (Vinca rosea) by dodder (Cuscuta campestris) (Garnier and Bové, 1983). Transmission of the greening-associated bacterium to periwinkle by grafting and Trioza erytreae were also reported (Pandey, 1986). The ultrastructural similarity of the organism in citrus and periwinkle as well as the identical sypmtoms expressed under different temperature conditions (Garnier and Bové, 1983) were indicative of successful transmission. A large number of infected sieve tubes and the very high titres of the bacteria in the periwinkle suggested that it could be an ideal source for isolation. However, all attempts to isolate the greening agent from this material failed (Garnier and Bové, 1983; Garnier et al., 1987).

Isolation attempts by Sibara (1982) using medium based on the components of phloem exudates and insect tissue culture formulations were more successful. This medium is known as MIG (Medium for the Isolation of Greening). A bacterium believed to be the greening-associated bacterium in South Africa was isolated in pure culture.

Subsequent isolations carried out by Duncan (1985) using the MIG medium and South African greening affected tissue collected from different areas, resulted in the accumulation of a further 5 isolates associated with the disease. These isolates were structurally similar to the organisms described in situ by Moll and Martin (1974).

Bacteria were also isolated from symptomatic leaves collected from greening affected areas in South Africa (Garnett,1985). Morphologically, the cultured organisms were similar to those described in the phloem cells and vector haemolymphs, measuring 3300 - 4400nm long and 300 - 450nm wide, with the characteristic cell wall morphology (Moll and Martin, 1974). Based on scanning electron microscopy these cells divide asymmetrical, possibly by budding (Ariovich

and Garnett, 1985; Moll and Martin, 1974; Naidu, 1981). Furthermore, the long thin rods occur along the surface of the colony and larger round structures occur in the centre, older part of the colony. Both forms occur in infected phloem sieve tubes and the psyllid haemolymphs (Chen et al., 1973; Garnier et al., 1976; Garnier and Bové, 1983).

Other isolation attempts (Garnier and Bové, 1983; Manicom, 1984; Garnier et al., 1987; Korsten et al., 1989) failed to obtain the greening-associated bacterium in pure culture. Previous reports on the isolation of a mycoplasma in pure culture believed to be the greening pathogen were made (Ghosh et al. 1971). Consequently, the credibility in the reported isolations of the greening-associated bacterium by Sibara (1982), Duncan (1985), Garnett (1984) and Mochaba (1988) are questionable. Clearly, the similarity in ultrastructure between the isolated cultures and the structures in vivo are too close to be disregarded.

By optimizing the isolation procedure, the bacterium associated with South African greening has been repeatedly isolated in pure culture (Mochaba, 1988). This bacterium was furthermore not observed in isolations performed simultaneously on healthy citrus tissues (Mochaba, 1988). Isolates have also been obtained from Reunion greening and Taiwan likubin maintained in planta at the Beltsville Agricultural Research Centre, Maryland, U.S.A. (Lee et al., 1989). Reinfection of healthy seedlings with the available isolates were performed to fulfil Koch's postulates. Subsequent characteristic greening symptoms developed. In several cases, the bacterium was re-isolated and re-identified as the original isolate (Mochaba, 1988; H.M. Garnett, personal communication) further supporting the claims that the bacterium isolated is a pathogen and closely associated with the disease.

1.7 Serological characterization

An antiserum was raised against the cultured organism (Sibara, 1982). Detection of greening-affected citrus preparations was obtained with the raised antiserum although the antibody titre was too low for these observations to be conclusive.

By optimizing the immunogen preparation (Duncan, 1985), an antiserum was available that could detect low concentrations of the cultured organism in an enzyme linked immunosorbent assay (ELISA). New isolates could be detected that were antigenicaly related to the greening-associated bacteria isolated from different areas in South Africa. Greening-affected citrus was readily distinguished from citrus affected by other diseases (Duncan, 1985; Duncan and Garnett, 1985).

Antisera have been prepared against several bacteria isolated from South African greening-affected citrus and other *in planta* sources in Beltsville (Mochaba, 1988; Garnett, 1985; Lee *et al.*, 1989).

Dot immunoassays have successfully been used to screen affected citrus samples from several countries (H.M. Garnett, personal communication). Preliminary observations suggest a serological relatedness between the cultured organisms from these areas (Garnett *et al.*, 1989) supporting previous observations (Duncan, 1985; Mochaba, 1988).

Attempts to use gold labelled antibodies raised against the cultured greening-associated bacterium were successful (Ariovich and Garnett, 1989). The gold-IgG probe reacted with both the long thin rods and round forms in petiole sections and crude extracts. Labelling of healthy tissue, *Bacillus subtilis*, *Erwinia liquefaciens* and *Escherichia coli* control cultures was not observed. Furthermore, the serological similarity between the cultured organism and the bacterium in greening

affected citrus led Ariovich and Garnett (1989) to conclude that the cultured organism is in fact the pathogen.

Parallel serological investigations were carried out by Matrin-Gros et al. (1987) and Garnier et al. (1987). Two monoclonal antibodies (10A6 and 2D12) have reportedly been raised against a greening-infected periwinkle immunogen. These monoclonal antibodies react specifically with preparations from both periwinkle and citrus infected with an Indian greening strain.

However, dot immunoassays and western blots using both monoclonal antibodies, 10A6 and 2D12, did not react with the greening bacterium cultured in vitro and with citrus samples from the in planta sources at Beltsville (unpublished results).

Using monoclonal antibodies, the greening-associated organism has been purified from infected periwinkle tissue and viewed under the electron microscope (Garnier et al., 1989). Both filamentous forms, 1.0 - 4.5µm long and 0.1 - 0.3µm wide, and round forms with a diameter of 0.2 - 1.5µm were observed. Different serotypes within strains of the greening organisms found in India, Indonesia and the Philippines have been reported (Garnier et al. 1989).

Monoclonal antibodies have furthermore been raised against Acinetobacter lwoffi isolated from greening affected citrus (Smith et al., 1988). These monoclonals have been utilized to screen plant and insect samples by ELISA, complement fixation and immunofluorescent microscopy. Consistent positive reactions with preparations of South African greening-affected citrus or preparations of T. erytreae taken from symptomatic plants was not obtained. Positive reactions were, however, reported with Asian greening from the Philippines. Monoclonal antibodies have also been raised against both greening diseased and healthy T. erytreae extracts. There were significant differences between the two sera in their ability to

detect greening positive and greening negative T. erytreae and diseased and healthy citrus columella extracts in dot blots and ELISA's (Korsten et al., 1989).

Polyclonal antibodies were raised to a partially purified bacterial preparation from affected citrus (Chippindall and Whitlock, 1989[1]). The successful use of this antiserum to detect greening in situ using ELISA and immunoblot assays is not clear (Chippindall and Whitlock, 1989[2]).

1.8 Identification

The identification of the bacterium associated with citrus greening disease is inconclusive.

Based on some unique properties of the bacterium isolated from greening-affected citrus, the organism was closely related to the genus Arthrobacter (Sibara, 1982). However, the bacterium was also similar to some Coryneform bacteria (Sibara, 1982).

Based on an Analytical Profile Index (API) characterization, the low temperature isolates are related to Agrobacterium radiobacter (Mochaba, 1988). Conventional biochemical tests and DNA hybridization studies however, did not support this. Spore formation in some isolates (Mochaba, 1988; Ariovich and Garnett, 1985) and an asymmetrical mode of division, further complicates the taxonomic placing of the bacterium.

A bacterium, identified as Acinetobacter lwoffi, has also consistently been isolated from greening affected citrus and found to induce greening-like symptoms after artificial transmissions (Smith et al., 1988).

Successful isolation of a bacterium from affected citrus, symptom development upon re-infection and subsequent re-isolation of the same bacterium has been carried out by Chippindall and Whitlock (1989[2]). However, psylla transmission could not be achieved and antisera raised against this bacterium failed to react with preparations of greening affected material. The organism was identified as a Clavibacter sp. and concluded to be a component of the disease syndrome.

Indeed, affected citrus trees may be infected with more than one organism each of which may influence symptom expression and the extent of the disease (da Graça, in press). In an attempt to clarify the identity the greening agent it consequently becomes important to identify the other agents that may naturally occur on healthy citrus and/or cause other citrus infections. The association of a Clavibacter sp., which is a Gram-positive non-spore former (Collins and Bradbury, 1986), with the greening disease syndrome can then be further understood.

1.9 The Australian situation

1.9.1 The Australian citrus industry

Citrus was first introduced to Australia in 1789 probably as seeds from Brazil and South Africa (Walpole, 1978). Because of the very dry Australian climate, orchards were at first restricted to the N.S.W. central coastal region. With the establishment of irrigation schemes along the Murrumbidgee and Murray rivers, the citrus industry spread inland in the early 1900's (Duncan, 1978; Walpole, 1978). Today citrus is grown in the Murrumbidgee irrigation area (MIA), the N.S.W. and Victorian Murray river areas, South Australia, Western Australia and Queensland.

1.9.2 Australian Citrus Dieback (ACD)

The literature on Australian citrus dieback is limited. Two papers by Broadbent et al. (1977[1+2]), have been published on the subject and occasionally brief referrals to dieback in Australia are made by other authors.

A disease similar to greening, Indian dieback, leaf mottle and likubin was observed in a single smooth sour orange tree along the Lower Murray in 1942. Between 1942 and 1968, a similar disease affected grapefruit and orange trees in the MIA and Murray river areas. By 1975, there was a marked increase in the number of dieback affected trees in the inland orchards. The incidence was, however, quite variable as some areas were hardly affected while other orchards had disease incidence rates of up to 80%. Grapefruit and smooth sour oranges were found to be most affected.

1.9.3 Symptomology

ACD symptoms resemble those caused by root rot, severe stem pitting, nutrient deficiencies and unthriftiness caused by nematode infection. The range and severity of the symptoms vary with the seasons, citrus varieties, tree age and nutrition. Symptom expression was strongest in grapefruit and sour orange during the autumn months. Symptoms often occur on single branches but then spread throughout the tree. The rate of spread of visible sypmtoms is greater in younger trees. During the summer months, symptom expression is greatly reduced.

1.9.3.1 Leaves

Affected leaves exhibit a variety of symptoms (Broadbent et al., 1977[1]). Patterns include yellowing of the midrib to complete chlorosis with green midveins and

lateral veins or green blotches and spots on a yellow leaf blade. These symptoms occur on grapefruit and to a lesser extent on sweet orange. New leaves on affected trees are narrower and shorter than normal and pale green to yellow in colour. Premature leaf drop also occurs.

1.9.3.2 Fruit

Affected grapefruit are smaller on diseased than on non-affected trees. In sweet oranges little variation in size occurs. The seeds of smooth sour oranges are often aborted.

1.9.4 Transmission

1.9.4.1 Graft transmission

Attempts to transmit the causal agent of dieback to healthy seedlings by grafting have generally been very successful. More severe symptoms appeared on glasshouse seedlings in winter than in summer. Under controlled conditions, symptoms developed better at 22°C - 25°C than at 28°C - 32°C. Transmission rates were higher when field material was collected during the spring and autumn months (Broadbent et al., 1977[1]).

1.9.4.2 Insect transmission

Neither Diaphorina citri nor Trioza erytreae occur in Australia. The Australian psyllid species on native vegetation are not regarded as citrus pests. However, in some cases psyllids move into citrus orchards from neighbouring native vegetation and consequently may have attributed to the spread of the pathogen (P. Barkley, personal communication). Several species of flattids, cicadellids and psyllids,

which are under investigation as potential vectors, have been collected in grapefruit groves.

1.9.5 A causal agent

Mycoplasma-like organisms occur in the vascular tissues of affected leaves. Observations were, however, limited to a single specimen and single sieve tube within that specimen. Attempts to isolate the ACD agent have been unsuccessful to date (Broadbent et al., 1977[1]).

1.9.6 Other

Gentisic glucoside occurrence in bark and albedo extracts of greening-affected citrus is a reliable indication of infection in South Africa (da Graça, in press) and in the in planta sources at Beltsville (R.F. Lee, personal communication). The phenolic compound has also been readily identified in Australian citrus dieback samples taken from trees that exhibit sypmtoms which closely resemble those of greening (Feldman and Hanks, 1968), although other diseased citrus yielded false positives (P. Barkley, personal communication).

1.10 Research objectives

Two distinct morphological forms of the bacterium associated with greening occur in the phloem sieve tubes, pysllid haemolymphs and in both plate and broth cultures of the isolated organism. The two forms are connected and plasmolytic effects may contribute to round form development (Garnier et al., 1976). The formation of round forms may be due to a nutritional stress (Ariovich and Garnett, 1985).

Assuming that the long thin rod and round form are indeed the same organism, it should be possible to establish a detection system, that recognises both morphological forms and the various reported strains of the greening-associated bacterium, based on the antigenic surface properties.

Based on the outer membrane and cellular proteins of cultures of the greening-asociated bacteria, separated by SDS-PAGE (Mochaba, 1988), protein pattern "fingerprints" are characteristic and useful for comparative studies. Indeed, this method has been used to differentiate Xanthomonas species (El-Sharkawy and Huisingh, 1971[2]), compare representatives of phytopathogenic bacterial genera (El-Sharkawy and Huisingh, 1971[1]), resolve the taxonomy of phytopathogenic Corynebacterium (Carlson and Vidaver, 1982) and identify citrus tristeza virus strains (Guerri et al., 1990).

The classification of the greening-associated bacterium has been unsuccessful although several suggestions have been made. Not only have many authors reported to have failed to culture the organism but once isolated the nutrient requirement in the sustaining medium is also very complex. Consequently, the use of conventional biochemical tests (Fahy and Hayward, 1983) and the commercially available Analytical Profile Index (API) systems have been inadequate (F. Mochaba, personal communication).

The objectives of this research were therefore:

a) - To establish the relationship between the "long thin form" and the "round form" of the greening-associated bacterium. Are they indeed morphological variations of the same organism changing shape with age and/or nutrient limitations or are the two forms representative of a close symbiotic relationship between different organisms?

b) - To raise antisera to representative putative greening-associated bacteria in culture isolated by Mochaba (1988), and determine the practicality of slot-blot immunobinding assays in the serodiagnosis and differentiation of the available putative greening-associated isolates from other Gram-negative laboratory cultures and plant pathogenic bacteria. Pending the success of these results, slot-blot immunoassays could then be further adapted to detect the greening pathogen in citrus suspected of infection.

c) - To obtain whole cell protein patterns by conventional electrophoretic methods and compare the resulting "fingerprints". Furthermore, by reacting the proteins with the raised antisera in western blots, the proteins associated with antigenicity can be determined and the resulting serological relatedness of the various isolates determined.

d) - To select the shared antigenic proteins and raise protein specific polyclonal antisera in an attempt to increase the specificity and sensitivity of detection in slot-blot immunoassays and western blots of bacterial isolate preparations.

e) - To determine the applicability of the serological detection techniques developed using <u>in vitro</u> cultures to differentiate between healthy and greening affected citrus preparations.

f) - To identify the cultured isolates using a recently developed method for the characterization and differentiation of Gram-negative aerobic bacteria - enteric, non fermenter and fastidious species (Bochner, 1989).

and

g) - To determine the similarity of Australian citrus dieback to greening and attempt to isolate the causal agent/s of ACD. Isolates are to be subjected to the morphological and serological comparative studies listed above.

2.0 MATERIALS AND METHODS

Unless otherwise specified, the chemicals used in the current study were obtained from AJAX Chemicals, N.S.W., Australia; BDH Chemicals Ltd., Dorset, U.K.; and/or MERCK, Darmstadt, Germany.

2.1 Bacterial cultures

Cultures of the putative citrus greening disease bacterial pathogen were obtained from successful isolations carried out by Mochaba (1988) and H.M. Garnett (Personal communication). These isolations were made from two sources:

a) Infected plant material collected from several areas in South Africa;

and b) The in planta culture collection maintained in Beltsville, Maryland, U.S.A., where plants have been grafted with original source material from most of the greening affected areas of the world.

Each isolate has been allocated a code to which it is referred in this research. Previous identities and code numbers are listed in Appendix I. All the isolates were obtained in complete liquid MIG medium and after subculture were able to pass through a 0.45µm filter and grow on solid MIG medium.

Note:

Actively growing greening-associated bacterial isolates obtained outside Australia were only researched at the University of the Witwatersrand, Johannesburg, South Africa and in the Agricultural Research Centre, Beltsville, Maryland, U.S.A. Only inactivated cultures were handled at the University of Wollongong in accordance

with the Australian quarantine regulations laid down by the Department of Agriculture. The inactivated cultures were obtained from stock cultures maintained at the Australian Animal Health Laboratory (A.A.H.L.) in Geelong, Victoria. These were grown in large volumes, inactivated and brought to the laboratory at Wollongong. Inactivated cultures, culture extracts and treated plant samples were also obtained from Beltsville, U.S.D.A.

Sexton tangerine and Madame Butterfly sweet orange seedlings and a Marsh grapefruit tree were available as sources of healthy plant material. The seedlings were grown from seed in a potting soil mixture containing a 1:2:1 peat moss, fine sand and pine mulch mixture supplemented with 1.7kg super phosphate, 2.25kg $MgCO_3$ and 1.0kg $CaCO_3$ (Adelaide & Wallaroo Fertilizers Ltd., S.A., Australia) in 180 x 350mm planter bags and watered with a solution containing per 20 litres: 22g $CuSO_4.5H_2O$, 9g $ZnSO_4$, 10g $MnSO_4$, 12g $FeSO_4.7H_2O$, 0.0625g $(NH_4)_6Mo_7O_{24}.4H_2O$ and 0.1875g H_3BO_3. The plants were maintained in the laboratory under ambient conditions. The grapefruit tree grew locally under natural conditions. Non-symptomatic leaf and fruit samples were also collected from the same orchards from which the greening and dieback samples were taken.

The South African isolates investigated were: SA01, SA02, SA03, SA05, SA06, SA07, SA09, SA10 and SA11.

Isolates from plant material originating in Reunion, RE01, and Taiwan, TA01 and TA02, were available, while American isolates included: US02, US03, US04, US05, US06, US07 and US08.

Isolates SA01 SA03, RE01, TA01 and TA02 were injected into healthy seedlings which subsequently developed symptoms similar to greening (Mochaba, 1988).

Bacteria suspected to be associated with the disease were subsequently isolated from plants infected with some of the isolates and were similar in many respects to the isolates originally injected (Mochaba, 1988). Inoculation of healthy seedlings with isolates SA01, RE01 and TA01 was also performed and characteristic greening symptoms observed (H.M. Garnett, personal communication).

Other bacterial strains included in this research were obtained from the Biological and Chemical Research Institute (BCRI), N.S.W. Department of Agriculture, Australia; the University of New South Wales (UNSW) culture collection and Wollongong University (WU), and include:

Agrobacterium radiobacter BCRI - DAR 30536

Bacillus subtilis UNSW - DAR 26772

Corynebacterium michiganense subsp. michiganense BCRI - DAR 41312
and BCRI - DAR 25721

Corynebacterium michiganense subsp. insidiosum BCRI - DAR 34839
and BCRI - DAR 26781

Escherichia coli UNSW - DAR 34854

Pseudomonas aeruginosa UNSW - DAR 41296

Pseudomonas cepacia BCRI - 26676

Pseudomonas diminuita BCRI - ATCC 19146

Pseudomonas fluorescens UW

Pseudomonas syringae pv. syringae BCRI - DAR 35680a

Salmonella typhimurium UNSW - M 206

A new genus was proposed for the plant pathogenic corynebacteria and consequently both Corynebacterium michiganense subsp. michiganense and Corynebacterium michiganense subsp. insidiosum were reclassified as Clavibacter michiganense subsp. michiganense and Clavibacter michiganense subsp. insidiosum (Collins and

Bradbury, 1986) abbreviated here to C. michiganense and C. insidiosum respectively.

2.2 Maintenance and storage

2.2.1 Cultures

All the isolates were periodically grown as plate cultures from which single colonies were transferred to McCartney bottles containing sterile dH_2O and stored at 10°C for up to 1 month. The plates were subsequently sealed with tape and stored at 4°C if required within 2 - 3 days.

For long term storage, the cultures were grown to log phase (approximately 24 - 36 hours) in complete MIG medium and freeze dried in ampoules which were sealed under vacuum and stored at room temperature. These cultures could be revitalised as needed by breaking open one end of the ampoule and resuspending the contents in 0.2ml of complete liquid medium. The 0.2ml suspension was then transferred to a larger volume of medium and incubated in a controlled temperature room at 25°C and/or 35°C with shaking at 150rpm (BIO-LINE orbital shaker 4200, Edwards Instrument Company, England).

2.2.2 Plant material

Leaf and fruit samples collected in the field were packed in plastic bags and kept on ice after collection and during transportation. Samples could if necessary, be stored in the plastic bags for up to 1 week at 4°C.

For long term storage, the midribs were removed from the leaves and cut into smaller pieces. The pieces were then placed in a screw cap tube with silica gel

pellets contained at the bottom of the tube by a cotton wool plug. The tubes were stored at 4°C and the silica gel periodically replaced as the tissue dried. When required, the midribs were rehydrated overnight in dH_2O at 4°C and blot dried before use. This method of storage was also adopted in the field for long transportation times.

2.3 Growth media

2.3.1 MIG (Medium for the Isolation of Putative Greening-associated bacteria)

MIG medium (Appendix II), first developed by Sibara (1982) and subsequently used by Duncan (1985) and Mochaba (1988), was used to propagate the greening isolates. Incomplete MIG medium (Mochaba, 1988) without foetal calf serum was used to grow cultures of the isolates for serological observations.

2.3.2 Other

Nutrient broth -NB (OXOID Ltd, Basingstoke, Hants., U.K.) was made up according to the manufacturers specifications.

Medium 523, pH 6.9 (Kado and Heskett, 1970) contained per litre: 10g sucrose, 8g casein hydrolysate, 4g yeast extract (OXOID Ltd, Basingstoke, Hants., U.K.), 2g K_2HPO_4 and 0.3g $MgSO_4.7H_2O$ in dH_2O and autoclaved at 121°C for 15 minutes.

Sucrose peptone agar (SPA) medium (Lelliott and Stead, 1987) was made by dissolving 10g sucrose and 10g peptone (OXOID Ltd, Basingstoke, Hants., U.K.) in dH_2O. After adjusting the pH to 7.4, the volume was made up to 1 litre and autoclaved at 121°C for 20 minutes.

Nutrient glucose agar (NGA) medium was made up by adding 1.0% (w/v) glucose to nutrient agar (OXOID Ltd, Basingstoke, Hants., U.K.) and further prepared according to the manufacturers specifications.

2.4 **Metabolic characterization** (Bochner, 1989)

Metabolic characterization was carried out using the Biolog GN microplate system (BIOLOG Inc, Hayward, California, U.S.A.) that more or less physiologically characterizes and differentiates some Gram-negative aerobic bacteria (enteric, non fermenter and fastidious species).

Colonies grown on complete MIG plates at 25°C for 18 hours were suspended in 0.85% NaCl, pH 7.0, and as per instructions the cell concentration adjusted to give an absorbance of 0.2 ± 0.05 at 590nm (Ultrospec II, LKB Biochrom, Cambridge, England). The microplate was then inoculated with 150µl of this bacterial suspension per well. The plates were incubated at 25°C for 24 hours and cell respiration recorded by the resulting reduction of the tetrazolium dye and purple colour development scored against a 0.85% NaCl control in well A1.

2.5 **Growth curves**

2.5.1 **Starter cultures**

Starter cultures were established by inoculating 10ml complete MIG medium in 100ml Erlenmeyer flasks with a loopful of a culture maintained either as a plate culture stored at 4°C or stored as a suspension in dH_2O at 10°C. The flasks were incubated on an orbital shaker (Certomat® R, B. Braun, Melsungen, A.G., W. Germany) at 150 rpm in a 25°C controlled temperature room and observed for growth. Turbidity became apparent within 2 - 7 days.

2.5.2 Growth cultures

Complete MIG medium (100ml), in 250ml Erlenmeyer flasks, was inoculated with 0.05 - 1.5ml of the above starter culture in early log phase to give a final bacterial concentration of 1 x 10^7 cells/ml. Replicate cultures (3 - 6) were incubated on orbital shakers (Certomat® R, B. Braun, Melsungen, A.G., W. Germany) at 150 rpm in both 25°C and 35°C controlled temperature rooms.

2.5.3 Sampling

Samples, 1.5ml, were taken at regular time intervals and fixed by adding 0.1ml 37% formaldehyde. After incubation for at least 4 hours at 4°C, the cells were centrifuged in an Eppendorf microfuge (Eppendorf Gerätebau, Netheler+Hinz, GmbH, Hamburg, W. Germany) at 3,000rpm for 10 minutes and resuspended in 1.5ml physiological saline (0.85% NaCl in dH_2O) containing 1.0% EDTA. The absorbance for each sample was recorded at 550nm on a Bausch and Lomb 1001 spectrophotometer (Bausch & Lomb Inc., Rochester, N.Y., U.S.A.), converted to give a 1/10 dilution reading (Appendix III) and graphed as a function of time.

2.6 Electron microscopy

2.6.1 Whole colony cross section preparation

Single colonies grown on complete MIG plates were cut from the medium and transferred into a glass tube (± 10mm in length and 5 mm diameter) containing 20% agar. After solidifying, the agar plug containing an intact colony was removed from the tube and trimmed. The colony was then fixed in 2.5% glutaraldehyde, washed twice in 0.1M Na-cacodylate buffer pH 7.2 and post fixed in 2.0% OsO_4. This was followed by staining overnight with a 1.0% aqueous solution of uranyl

acetate. The colony was then dehydrated through a 50% - 100% alcohol series and embedded in an Epon/Araldite mixture. Ultrathin sections (< 60nm) were cut on a Reichert ultramicrotome. The sections were subsequently stained with uranyl acetate followed by lead citrate and viewed on a JEM-100S transmission microscope (JEOL Ltd., Tokyo, Japan).

2.6.2 Negative staining

Size 300 mesh grids were coated with a parlodium (3.5% in amyl acetate) film to which a light carbon coat was applied. Morphological changes were observed by taking a drop from relevant cultures, applying this to the coated grid and blotting off the excess fluid with filter paper. The grid was subsequently inverted onto a drop of a 1% aqueous solution of uranyl acetate on parafilm and stained for 10 minutes. The grids were then rinsed with dH_2O to remove excess stain and allowed to air dry. The morphology of the bacterial cells was viewed in a JEM-100S electron microscope (JEOL Ltd., Tokyo, Japan).

2.7 Isolations

Samples affected with Australian citrus dieback (ACD) were collected from citrus orchards in the Murray river area (MA) in Victoria and the Murrumbidgee river irrigation area (MIA) of N.S.W. during the autumn, winter and early summer months. Marsh grapefruit leaf and fruit samples showing characteristic symptoms were collected from Gol Gol (MA) in April, 1987 and Nangiloc (MA) during July/August, 1987. Marsh grapefruit and Valencia orange leaf and fruit samples were collected from Griffith (MIA) in April, 1987. Marsh grapefruit leaf and fruit samples were again collected from Griffith (MIA) in September, 1990. Valencia orange samples were collected from Dareton (MA) during July/August, 1987. The samples were packed in plastic bags and transported as described in section 2.2.2.

The isolations were performed immediately upon arrival of the samples in the laboratory. In some cases the samples had to be stored at 4°C but were used for isolation attempts within a week.

The isolation procedures from leaf midribs and fruit columella (Mochaba, 1988) were aimed at obtaining bacteria morphologically similar to the putative greening-associated bacterium (Gram-negative small thin rods).

2.7.1 From leaves

About 5 - 6 midribs from infected leaf tissue, washed in dH_2O, were cut out and surface sterilized by wiping with 4% Na-hypochlorite. After soaking in 0.4% Na-hypochlorite for 10 minutes, the midribs were washed twice with sterile dH_2O and transferred to a petri-plate. To expose phloem limited bacteria, the midribs were finely chopped. A few drops of sterile water were added to assist emulsification. The fine mulch was then transferred to a test-tube containing 5ml sterile dH_2O and vortexed thoroughly. The midrib mulch was allowed to settle and 0.5ml of the supernate transferred to duplicate flasks containing complete MIG medium which were incubated at 25°C, 30°C and/or 35°C with shaking at 150rpm as above. The flasks were observed daily for indications of growth.

2.7.2 From fruit

Depending on the fruit size, 1 - 3 fruit were washed in dH_2O, the fleshy outer skin removed and the whole fruit wiped with 4% Na-hypochlorite. The washed area on top of the fruit was cleared by thinly slicing away the surface and the top of the core removed and transferred to a petri-plate. The fruit was then cut in half exposing the columella running down the core of each half. This columella was cut out and transferred to a petri-plate. A few drops of sterile water were added and the

columella chopped to a fine mulch. The fine mulch was then transferred to a test-tube containing 5ml sterile dH_2O and vortexed thoroughly. The columella mulch was allowed to settle and 0.5ml of the supernate transferred to duplicate flasks containing complete MIG medium which were incubated at 25°C, 30°C and/or 35°C with shaking at 150rpm as above. The flasks were observed daily for indications of growth.

2.7.3 Filter test for presumptive isolates

A 27mm diameter, 0.45µm pore size millipore filter (Millipore Corporation, Bedford, MA, U.S.A.) was placed on the surface of complete MIG in a petri plate. Approximately 10µl of a suspension of the putative greening isolate obtained from isolations as described above was placed on the filter and the plate incubated overnight at 25°C, 30°C and/or 35°C. The filter was then removed, discarded and the plate re-incubated. Bacteria less than 0.45µm in size would penetrate the filter and grow as colonies within 3 - 5 days.

2.8 Electrophoresis

2.8.1 Culture preparation for SDS-PAGE

Samples (10ml) of an early log phase growth culture (24hrs - 48hrs) grown in incomplete MIG medium were centrifuged at 5,000rpm (Sorvall RC5 centrifuge, Du Pont Instruments, Newtown, Conn., U.S.A.) for 15 minutes at 4°C. The pellets were suspended in 25ml 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV) and the cell concentration adjusted to an absorbance of 0.5 ± 0.01 at 550nm and 1cm light path (Ultrospec II, LKB Biochrom, Cambridge, England). The suspensions were further centrifuged at 5,000rpm for 30 minutes at 4°C and the pellets resuspended in 2.5ml PBS. After sonicating for 1 hour in a Bransonic B-12

sonicating ice bath (Branson Cleaning Equipment Company, Shelton, Conn., U.S.A.), 20µl aliquots of the sonicated suspension were removed for a Bio-Rad (Bio-Rad Laboratories, California, U.S.A.) protein estimation. The remaining suspensions were centrifuged in an eppendorf microfuge (Eppendorf Gerätebau, Netheler+Hinz, GmbH, Hamburg, W. Germany) at 12,000rpm for 10 minutes. The pellets were resuspended in 0.25ml PBS to which 0.75ml sample buffer, containing per 8ml: 4ml dH_2O, 1.0ml 0.5M tris-HCl, 0.8ml glycerol, 1.6ml 10% SDS, 0.4ml 2β-mercaptoethanol and 0.2ml 0.05% bromophenol blue, was added and heated at 100°C for 5 minutes. Aliquots containing 10 - 100µg total protein were loaded onto a 10% - 17.5% gradient gel system.

2.8.2 Gel preparation

The procedure for discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS) was adapted from that described by Laemmli (1970). A linear 10% - 17.5% polyacrylamide gradient separating gel (100mm long, 145mm wide and 0.75mm thick) and 4% stacking (upper) gel (20mm long, 145mm wide and 0.75mm thick) was used. Samples were electrophoresed in a 25mM Tris - 190mM glycine buffer, pH 8.3 - 8.6 containing 0.1% SDS. Electrophoresis was carried out at room temperature with a BWD 216A power supply (BWD Electronics Pty. Ltd., Melbourne, Australia). A constant voltage of 125V was applied until the bromophenol blue dye was eluted from the separating gel (about 6 - 6.5 hours). Proteins were visualized in 0.75mm thick gels stained overnight with 0.1% Coomassie blue R250 (SIGMA Chemical Co., St. Louis, MO, U.S.A.) in 50% dH_2O, 40% methanol and 10% glacial acetic acid. Gels were destained in solution containing 50% dH_2O, 40% methanol and 10% glacial acetic acid until the background was sufficiently reduced (usually 2 - 3 changes of the destaining solution). Proteins separated in 1.5mm thick gels were further treated as required for serological analyses (Ref. 2.9.7). Phosphorylase b

(Molecular weight 94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) trypsin inhibitor (20100) and α-lactalbumin (14400) (Pharmacia Inc., New Jersey, U.S.A.) were used as reference proteins.

2.9 Serological applications

2.9.1 Immunogen preparation

2.9.1.1 Whole cells

An overnight culture grown in incomplete liquid MIG medium was washed 3 times by low speed centrifugation, with 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV). The cells were suspended in PBS + 0.2% (v/v) formaldehyde at a concentration of 1 x 10^9 cells/ml. After standing at room temperature for a minimum of 4 hours, the bacterial cells were again washed 3 times in PBS as before and prepared for intra-venous (IV) and intra-muscular (IM) immunizations as described by Duncan (1985) and E.L. Civerolo (Personal communication).

For IV immunizations, the cells were either adjusted as described by Duncan (1985), to a concentration of 1 x 10^7 cells/ml in PBS + 50% (v/v) incomplete Freund's adjuvant (SIGMA Chemical Company, St. Louis, MO, U.S.A.) or diluted with 2 volumes of PBS resulting in a net cell concentration of 3.3 x 10^8 cells/ml (E.L. Civerolo, personal communication). Both preparations were stored at 4^oC until used.

For IM immunizations, the cells were either adjusted as described by Duncan (1985), to a concentration of 1 x 10^9 cells/ml and homogenised with an equal volume of complete Freund's adjuvant (SIGMA Chemical Company, St. Louis, MO, U.S.A.) resulting in a cell concentration of 5 x 10^8 cells/ml or diluted by adding an

equal volume of PBS and homogenised with an equal volume of incomplete Freund's adjuvant (E.L. Civerolo, personal communication) resulting in a cell concentration of 2.5 x 10^8 cells/ml. Both preparations were stored at 4^0C until used.

2.9.1.2 Gel piece

Fifteen wells in SDS-PAGE gels, were each loaded with 50µl of culture (approximately 35µg of protein per well) and prepared as described in section 2.8.1. Duplicate gels (0.75mm thick slabs) run according to the procedure described in section 2.8.2 were stained overnight with an aqueous Gradipure gel stain, a ready to use colloidal form of Coomassie blue G-250 (GRADIPORE Ltd., Pyrmont, Australia), and rinsed with several changes of dH_2O. Desired protein bands were cut from each lane and the gel pieces transferred to the barrel of a 2ml syringe and forced through the outlet into a second 2ml syringe. This was repeated several times to fragment the gel pieces. A connection was then introduced between the outlets of the two syringes using two 15G needles joined and encased by a piece of tight fitting tubing forming a double-hub system. To facilitate the emulsion of the gel, 1.2ml 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV) was added and the pieces repeatedly passed through the 15G double-hub. This emulsion was then made even finer using a 23G double-hub followed by a 25G double-hub system. An equal volume of complete Freund's adjuvant (SIGMA Chemical Company, St. Louis, MO, U.S.A.) was added and the suspension thoroughly mixed using the 23G double-hub. The suspension was then split equally into each syringe (approximately 1ml in each) and stored at 4^0C.

New Zealand white rabbits were used to raise antisera and immunized according to the schedule illustrated in Table 1. All IV immunizations were made into the marginal vein of the ear while IM immunizations were made into the thigh muscles.

TABLE 1 Immunization schedule

Antisera were raised in New Zealand white rabbits against protein-containing gel homogenates and SA01, SA03, SA07 and RE01 whole cell preparations as indicated. Intra-venous (IV) immunizations containing incomplete Freund's adjuvant and intra-muscular (IM) immunizations containing complete Freund's adjuvant were generally given although in some cases SA01 cell preparations without adjuvant were injected intravenously (IV-).

	ISOLATE				
DAY	SA01	SA03	SA07	RE01	GEL
0	0.75ml (IV-)	0.5ml (IV)	1.0ml (IV)	0.4ml (IV)	1.0ml (IM)
2		0.5ml (IV)	1.0ml (IV)	0.5ml (IV)	
3					1.0ml (IM)
4	0.75ml (IV-)	0.5ml (IV)		0.5ml (IV)	
5			0.2ml (IV)		
6	1.0ml (IM)	0.5ml (IV)		0.4ml (IV)	1.0ml (IM)
7			0.2ml (IV)		
8		0.5ml (IV)		0.5ml (IV)	
9			0.2ml (IV)		1.0ml (IM)
10		0.5ml (IV)		1.0ml (IM)	
11			0.2ml (IV)		
12		0.5ml (IV)		1.0ml (IM)	1.0ml (IM)
13	1.0ml (IM)		0.2ml (IV)		
14				1.0ml (IM)	
15		Test Bleed			1.0ml (IM)
16	Test Bleed			1.0ml (IM)	
18		0.5ml (IV)			1.0ml (IM)
19	0.75ml (IV-)		Test Bleed		
21		Test Bleed	0.5ml (IV)	0.5ml (IM)	
22	Test Bleed				
23		Total Bleed			
24	Total Bleed				
25				Test Bleed	
26			Test Bleed		
27				0.5ml (IV)	
29				0.5ml (IV)	
30					1.0ml (IM)
31				Total Bleed	
32			Total Bleed		
36					Test Bleed
38					Total Bleed

2.9.2 Serum from blood

Test bleeds consisted of 5 - 10ml of blood taken from the marginal ear vein. Total bleeds were performed by heart puncture yielding a volume of blood of 60 - 115ml depending on the size of the rabbit and the success of the bleed. The blood was left to coagulate at 37°C for 4 hours upon which it was stored overnight at 4°C. The serum was pipetted off, centrifuged at 2,000rpm in a Beckman TJ6 centrifuge (Beckman Instruments Inc., Palo Alto, California, U.S.A.) for 10 minutes and the supernate aliquotted into 1 - 2ml volumes. Aliquots were stored at -20°C.

The antibody titres in the test bleed samples from animals immunized with whole bacterial cells were determined by reacting the crude antiserum with the corresponding antigen in a slot-blot immunoassay (Ref. 2.9.6.1). Two-fold dilutions in 0.15M phosphate buffered saline (PBS), pH 7.2 (Appendix IV) of the test bleed serum was reacted with ten-fold dilutions in PBS of the corresponding formaldehyde fixed bacteria in a checkerboard arrangement. An antibody dilution of 1:1,024 or better was required before the total bleed was performed.

Similarly the serum in the test bleeds from animals immunized with protein bands was screened for the ability to react with the relevant band in a western blot (Ref. 2.9.7). The reaction of the protein bands to which antisera were raised was scored as either positive or negative.

2.9.3 IgG Purification

2.9.3.1 **Ammonium sulphate precipitation** (Hudson and Hay, 1980)

The serum was diluted with two volumes of physiological saline and then saturated ammonium sulphate solution was added to give a final concentration of 45% (v/v).

The mixture was stirred at room temperature for 1 hour. The precipitate was collected by centrifuging at 3,000rpm (Sorvall RC5 centrifuge, Du Pont Instruments, Newtown, Conn., U.S.A.) for 30 minutes and washed once with 45% (v/v) saturated ammonium sulphate solution in saline. The pellet was resuspended in a minimal volume of 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV) and centrifuged at 1,000rpm for 45 minutes to remove insoluble material. Excess ammonium sulphate was removed by dialyzing overnight against 5 changes of 1litre volumes of PBS at 4°C. The partially purified immunoglobulins were aliquotted into 1ml volumes and stored at -20°C.

2.9.3.2 Affinity chromatography

The IgG fraction was further purified from the partially purified immunoglobulin preparation (section 2.9.3.1) by affinity chromatography on a DEAE AFFI-GEL Blue column (Bio-Rad Laboratories, California, U.S.A.). The IgG containing fractions were eluted from the column with 0.02M Tris-HCl, 0.028M NaCl, pH 8.0 containing 0.02% NaN_3 and pooled. The chromatographically purified IgG was concentrated in Centriprep-10 concentrators (Amicon, W.R. & Co., Massachusetts, U.S.A.), aliquoted into 1ml volumes at a concentration of 1mg/ml and stored at 4°C. NaN_3, 0.01%, was added as a preservative.

2.9.4 Other antisera

Antisera 228/12, 228/13, 228/14, 228/15, 228/16 and 228/17 raised in the U.S.A. were also available and used in western blots. These antisera were raised to protein bands in the high 30K - low 40K molecular weight range that reacted with UF6 antiserum in western blots of samples prepared from greening affected tissue. UF6 antiserum was raised in the same laboratory against fixed GL isolate (identified in this research as SA01) whole cells (R.F. Lee, personal communication).

2.9.5 Cross-absorption

2.9.5.1 With bacteria

Bacteria were grown in either incomplete MIG medium (putative greening-associated isolates) or NB (other cultures) at 25°C and the cell concentration determined using a haemocytometer as soon as the cultures appeared slightly turbid (usually within 24 hours of growth when an actively growing culture was used as an inoculum). The volume required to establish a cell concentration of 5×10^9 cells/ml of the bacteria in a 25ml total volume was then transferred to a 30ml centrifuge tube and the remaining volume made up to 25ml with antibody buffer containing 1% gelatin in TBS (20mM Tris, 500mM NaCl and 0.02% NaN_3 in dH_2O, pH 7.5). Purified IgG at 1mg/ml, 25µl, was added and the tube left to incubate for 1 hour at 37°C with shaking (Orbital shaker incubator 461, Paton Industries Pty. Ltd., Stepney, S.A., Australia). The cells and cross-absorbed antiserum/-a were removed by centrifuging at 11,000rpm (Sorvall RC5 centrifuge, Du Pont Instruments, Newtown, Conn., U.S.A.) for 10 minutes and the supernatant stored at 4°C until used.

2.9.5.2 With plant material

Healthy Marsh grapefruit midribs were homogenized on ice in 0.15M phosphate buffered saline (PBS), pH 7.2, 1:5 (w/v) (Appendix IV) using a polytron (Kinematica, Switzerland) set at 3 for 3 x 5 second bursts with 1 minute cooling intervals. The homogenate was filtered through a single layer of Miracloth® (Chicopee Mills Inc., Milltown, NJ, U.S.A.). The filtrate (2ml) was added to 23ml antibody buffer containing affinity purified IgG at a concentration of 10 µg/ml. After incubating at 37°C for 1 hour, the cross absorbed complexes were removed by centrifuging at 11,000rpm (Sorvall RC5 centrifuge, Du Pont Instruments,

Newtown, Conn., U.S.A.) for 10 minutes and the supernatant stored at 4°C until used.

2.9.5.3 With LPS

Purified IgG was cross absorbed with LPS (DIFCO, Detroit, U.S.A., 2mg/ml stock solution) by incubating 25µl of the purified IgG preparation with 3ml of LPS stock solution in 22ml antibody buffer and incubated at 37°C for 1 hour with shaking (Orbital shaker incubator 461, Paton Industries Pty. Ltd., Stepney, S.A., Australia). The unbound LPS and cross absorbed antibodies were then removed by centrifuging at 11,000rpm (Sorvall RC5 centrifuge, Du Pont Instruments, Newtown, Conn., U.S.A.) for 10 minutes and the supernatant stored at 4°C until used.

2.9.6 Slot-blot immunoassays (R.F. Lee, personal communication)

2.9.6.1 Bacterial isolates

Nitrocellulose membrane (0.45µm pore size; Schleicher & Schuell Inc, New Hampshire, U.S.A.), was cut to fit a Minifold-II slot-blotter (Schleicher & Schuell Inc, New Hampshire, U.S.A.), prewet in TBS (20mM Tris, 500mM NaCl and 0.02% NaN_3 in dH_2O, pH 7.5) and assembled in the apparatus. Ten µl aliquots, (culture concentrations adjusted based on cell counts or absorbance of suspensions in 0.15M PBS, pH 7.2, as appropriate) were applied to the wells and the membrane air dried for 1 hour before soaking in 3% blocking solution (3g gelatin in 100ml TBS) with rocking (BIO-LINE Platform Rocker 4200, Edwards Instrument Company, England). Excess blocking solution was removed by washing 2 x 5 minutes with Tween-TBS (0.5ml Tween 20 per litre TBS). The membrane was then transferred to antibody buffer (1g gelatin per 100ml TBS) containing purified IgG at a

concentration of 10µg/ml and allowed to rock at room temperature for 12 hours. After 2 x 5 minute and 1 x 10 minute washes in TTBS, the membrane was soaked in goat anti-rabbit alkaline phosphatase (GAR-AP, Promega Corporation, Wisconsin, U.S.A.) diluted 1/7,500 in antibody buffer, allowed to rock at room temperature for 4 hours and washed 3 x 5 minutes in TTBS and 1 x 15 minutes in TBS.

The colour development solution (Promega Corporation, Wisconsin, U.S.A.) containing 33µl nitro blue tetrazolium (NBT) stock solution (50mg/ml in 70% dimethylformamide) and 16.5µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) stock solution (50mg/ml in dimethylformamide) in 5ml alkaline phosphatase buffer (100mM Tris-HCl, pH 9.5, 100mM NaCl and 5mM MgCl) was added and allowed to develop for 10 - 15 minutes. The reaction was stopped with dH_2O.

Preparation of the buffers is described in Appendix VI.

2.9.6.2 Modifications to culture preparations for slot-blots

Overnight cultures grown in incomplete MIG medium were washed 3 times in 0.15M phosphate buffered saline (PBS), pH 7.2, (Appendix IV) and the cells fixed in PBS + 0.2% (v/v) formaldehyde at a concentration of 1 x 10^9 cells/ml. After standing at room temperature for 4 hours the cells were washed 3 times in PBS adjusted to a concentration of 1 x 10^8 cells/ml and stored at 4°C.

The formaldehyde fixed cell preparation was also sonicated for 1 hour in a Bransonic B-12 sonicating ice bath (Branson cleaning equipment company, Shelton, Conn, U.S.A.) and the preparation stored at 4°C.

In addition to the two treatments mentioned above, the fixed and sonicated cell preparation was further centrifuged in an Eppendorf microfuge (Eppendorf

Gerätebau, Netheler+Hinz, GmbH, Hamburg, W. Germany) at 12,000rpm for 10 minutes and the pellet resuspended in an equal volume of splitting solution (containing per 8ml: 4ml dH_2O, 0.5M Tris-HCl, 0.8ml glycerol, 1.6ml 10% SDS and 0.4ml 2β-mercaptoethanol). The preparation was heated in a boiling water bath for 5 minutes and stored at 4°C.

2.9.6.3 Citrus tissue preparation for slot-blots

The procedure employed for the slot-blot immunoassays of affected citrus tissues is similar to that described for bacterial cultures (2.9.6.1) although a few modifications were incorporated.

Samples were prepared by homogenizing the midribs from symptomatic and non-symptomatic leaves in 0.15M phosphate buffered saline (PBS), pH 7.2, (Appendix IV) at a ratio of 1:10 (w/v). The homogenate was then sonicated in a sonicating ice bath (Branson Cleaning Equipment Company, Shelton, Conn., U.S.A.) for 1 hour and filtered through a single layer of Miracloth® (Chicopee Mills Inc., Milltown, NJ, U.S.A.). Aliquots (10µl) of the filtrate were applied to the slot-blotter.

The use of the GAR-AP system of detection (Ref. 2.9.6.1) can be applied to citrus tissue assays even though care must be given to discount any interference caused by the green pigments when analysing the results. For confirmation, ^{125}I labelled donkey anti-rabbit (Amersham Laboratories, Buckinghamshire, U.K.) second antibody, diluted 1/500, in the antibody buffer was used and allowed to rock for 4 hours. After exposure to this reagent, the membrane was washed 6 x 10 minutes in TTBS followed by 3 x 10 minute washes in TBS and allowed to air dry overnight before exposing the membrane to autoradiography film (Hyperfilm-MP, Amersham Laboratories, Buckinghamshire, U.K.) for 21 days at -80°C.

2.9.6.4 Citrus tissue preparation for western blots

Samples were prepared by homogenizing the midribs from symptomatic and non-symptomatic leaves in 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV) at a ratio of 1:5 (w/v) and/or 1:10 (w/v). Aliquots of the homogenate, 2.5ml, were sonicated in a sonicating ice bath (Branson Cleaning Equipment Company, Shelton, Conn., U.S.A.) for 1 hour and filtered through a single layer of Miracloth® (Chicopee Mills Inc., Milltown, NJ, U.S.A.) and further treated as described in 2.8 and 2.9.7.

2.9.6.5 Preparation of phloem exudates from citrus tissue for western blot analysis (R.F. Lee, personal communication)

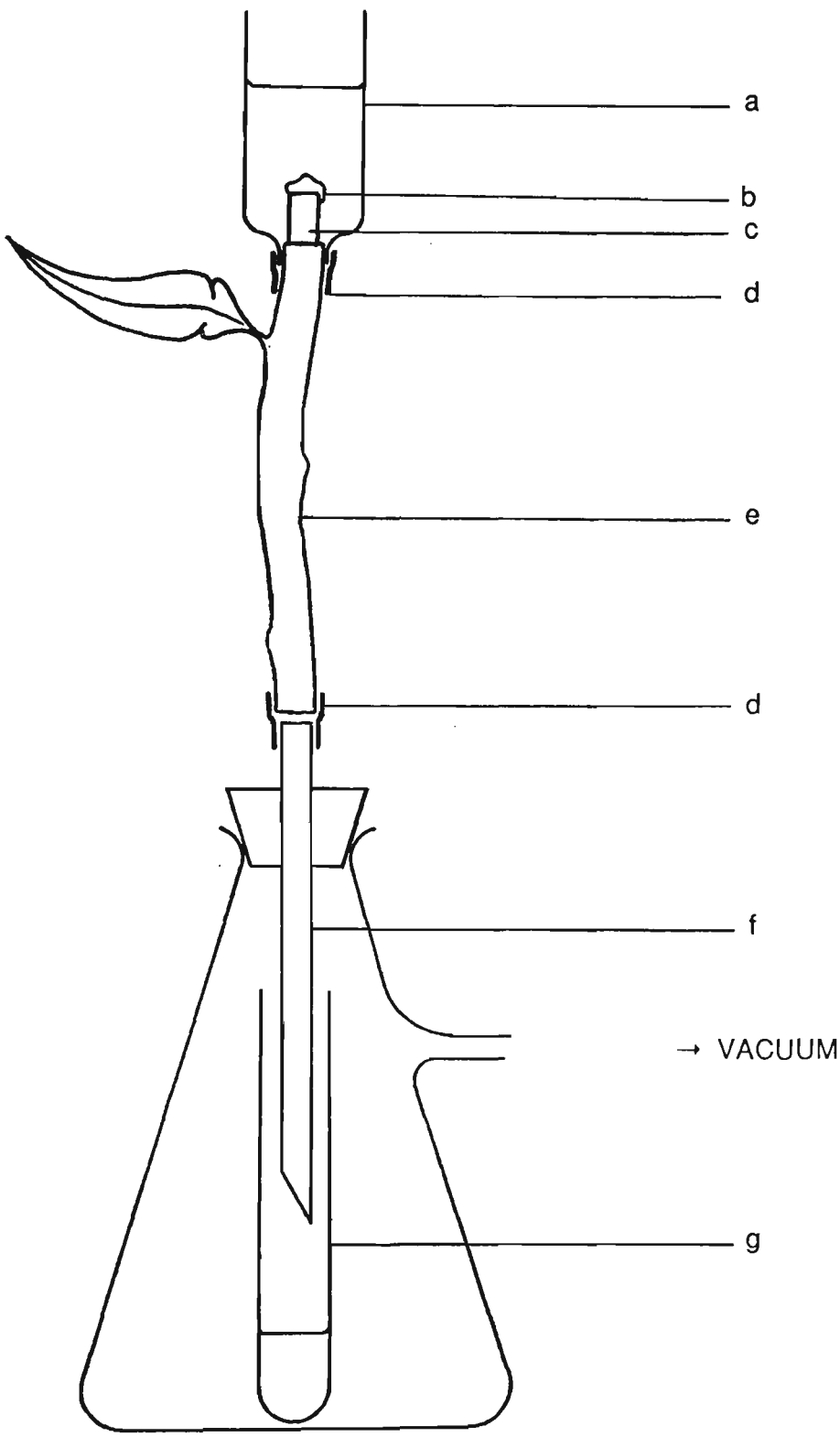
Twigs, approximately 5mm in diameter, were collected in the same orchards from greening/dieback affected trees and non symptomatic trees and cut into 10cm lengths. About 1 - 1.5cm of the bark was removed from one end and the xylem at this end plugged with wax. The twig was then attached to a vacuum flask as illustrated in Figure 1. Vacuum extraction buffer (0.05M Tris, 0.1% cysteine, 0.1% ascorbic acid and 0.5% 2β-mercaptoethanol, pH 8.0 in dH_2O), 2ml/10cm lengths, was pulled through and mixed 3:1 (v/v) with sample buffer (0.8g SDS, 4.0ml glycerol, 2.0ml 2β-mercaptoethanol and 2.5ml 1.0M Tris-HCl pH 6.8, diluted to 10ml with dH_2O). The samples were boiled and further treated as described in sections 2.8 and 2.9.7.

2.9.7 Western blots (Beisiegel, 1986)

Proteins separated by SDS-PAGE were transferred to nitrocellulose using the Multiphor II novablot system 2117 (Pharmacia LKB Biotechnology AB, Bromma, Sweden).

FIGURE 1 Assembly for phloem extraction from affected plants

The vacuum extraction buffer was applied to a resevoir (a) connected to the twig (e) by means of a piece of tight fitting rubber tubing (d). The bark at the top end (approximately 1cm) of the twig was removed (c) so that the phloem vessels were not blocked when wax (b) was applied to block the xylem vessels. The bottom of the twig was connected by means of a piece of tight fitting rubber tubing (d) to a glass tube (f), the other end of which ended in a collection tube (g). Both the glass tube and the collection tube were placed in a flask to which a vacuum was applied. The vacuum extraction buffer was pulled through the twig and the phloem exudate collected.


a
b
c
d
e
d
f
→ VACUUM
g

Nine sheets of blotting paper (Pharmacia LKB Biotechnology AB, Bromma, Sweden) trimmed to fit the slab gel, were soaked in anode buffer (48mM Tris, 39mM glycine in dH_2O and 20% methanol) and layered onto the anode plate saturated in dH_2O. The nitrocellulose membrane also soaked in the anode buffer was then layered onto the blotting papers. The slab gel (1.5mm thick) was then layered onto the nitrocellulose. A further 9 sheets of blotting paper soaked in cathode buffer (48mM Tris, 39mM glycine and 0.0375% SDS in dH_2O) were then layered onto the slab gel and the cathode plate saturated with dH_2O placed on top of these.

Protein transfers were carried out at a constant current of 0.64 mA/cm^2 for 2.5 hours after which the nitrocellulose membrane was removed from the apparatus, allowed to air dry for 1 hour and then treated as described in 2.9.6.

The LMW markers were cut from the membrane before blotting and visualized by staining with 0.01% amido black in 50% dH_2O, 40% methanol and 10% acetic acid for 10 minutes. The markers were destained with 50% dH_2O, 40% methanol and 10% acetic acid for 2 x 1 hour followed by 1 x overnight with rocking (BIO-LINE platform rocker 4100, Edwards Instrument Company, England).

2.10 Statistical analysis

Dr K.Russell of the Statistics Department, University of Wollongong kindly provided advice on the handling and analysis of the data.

To compare the exponential growth rate of the batch cultures at the 25°C and 35°C temperatures, a statistical analysis based on the straight line equation $y = mx + c$ was carried out where (m) represents the slope of the exponential phases. Any temperature dependant variations in the stationary phase bacterial titres identified

by the y-intercept (c) were also analysed by the same approach. Two hypotheses were consequently tested:

a) The exponential phases at 25°C and 35°C have a common slope i.e. the growth rate is the same at both temperatures.

b) There is no difference in the y-intercepts of the stationary phase curves of both temperatures.

The lines fitted to the stationary phase data for the two temperatures were accordingly tested for the same slope and if they could be regarded to have the same slope, was this common slope equal to zero? Where the slopes were significantly different from one another, either line was tested to see if it could be regarded as horizontal.

From the growth curve plots, the exponential and stationary phase regions to be investigated were determined for each culture. The required data for these regions included the absorbance values for each of the replicate curves, corresponding times post inoculation and the temperature of growth, either 25°C or 35°C.

An analysis of variance (ANOVA), including histograms of residuals and plots of the residuals against the predicted (or "fitted") values; the y-values predicted by the straight lines, was carried out on the data.

Conclusions were drawn from the F-statistic in relation to calculated p-values representing the probability of getting a value larger than the one observed by chance alone at the 5% level of significance.

2.11 Molecular weight determinations

Standard curves were established for gradient gel and western blot results by plotting the logarithms of the low molecular weights (LMW) markers against the distance travelled in mm.

Because the staining procedure for the western blot markers causes a slight shrinking in the supporting membrane with respect to the remaining blot, a conversion factor relating the LMW membrane to that of the remaining membrane had to be calculated for each western blot. Dividing the blot membrane length by the LMW marker membrane length gives the required conversion factor accounting for the difference between the lengths. The distances travelled for each of the markers multiplied by this conversion were used to plot the standard curves to which any observations on the western blots could be related.

In addition to the 94K - 14.4K standard curve two further plots were included. A plot for the 94K, 67K and 43K markers and another for the 43K, 30K, 20.1K and 14.4K markers (Appendix IX). Calculations of the molecular weights for unknown proteins were more accurate and consistent using the latter curves. The appropriate plot was chosen as required. The molecular weights of proteins travelling 30 ± 5mm were calculated from both the 94k - 43K and 43K - 14.4K standard curves. The average of the two assigned molecular weights was taken as the representative molecular weight for that protein.

3.0 RESULTS

3.1 General characteristics

3.1.1 Light microscope observations

In Gram stain smears of the available putative greening-associated isolates grown in complete MIG (Section 2.3.1) liquid and plate cultures, the bacterial cells were predominantly small, thin, rods of the Gram-negative type.

Smears of cells grown in complete liquid MIG medium and stained directly without first washing the cells, appeared Gram-positive. This is apparently due to the presence of media constituents in the smear. Smears made of cells grown in complete liquid medium also contained Gram-negative staining bulbous structures often mistaken for the round form of the presumptive greening-associated bacterium. These structures were serum globules formed during heat fixing. Neither of these problems occurred with cells from cultures grown in incomplete MIG broth or on complete/incomplete MIG plates.

Cells of isolates SA01, SA03, SA05, SA06, SA11, RE01, TA01, TA02 and US04 characteristically appear as small, thin, irregular rods (Figure 2a) and may vary in length from relatively long cells (Figure 3a) to very short cells (Figure 4a). Filament formation was rarely observed in these cultures. SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 adopt a more long, thin rod-shaped morphology generally varying only slightly in thickness and length (Figure 5a). Although the cells of isolate SA07 appear as a long thin rods in young cultures, the cells lengthen considerably with age. Indeed, the cells of a two week old SA07 culture can become entirely filamentous. What appear to be subterminal spore-like structures (Figure 6a) occurred regularly in both old liquid and plate cultures

of isolate SA07 and occasionally in cultures of isolates SA09, US02, US05, US07 and US08.

After 5 - 6 passages on either complete MIG plates or in complete MIG broth, all the isolates were able to grow on alternative media (Section 2.3.2) such as nutrient agar (NA), nutrient glucose agar (NGA) and 523 medium. The morphology of cells of isolates SA01, SA03, SA05, SA06, SA11, RE01, TA01, TA02, and US04 differed when grown on these media. Instead of the long, thin, rod morphology when grow on MIG, the cells were very much smaller and more oval in shape. The SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 cells appeared rounder, fatter and had a more granular interior compared to the cells grow on MIG. The spore-like structures associated with the cells of isolates SA07, SA09, US02, US05, US07 and US08 in MIG, also occurred when the isolates were grown on alternative media.

A further morphological variation that occurred in all the isolates, was the sporadic development of large bulbous structures extending from the cells (Figure 7) that were distinctly different from serum globules. Although these extensions occurred largely at the terminal ends of the cells resulting in dumb-bell and club-shaped structures, occasionally the centre of the cell bulged.

3.1.2 Plate culture observations

3.1.2.1 Colony morphology and pigmentation

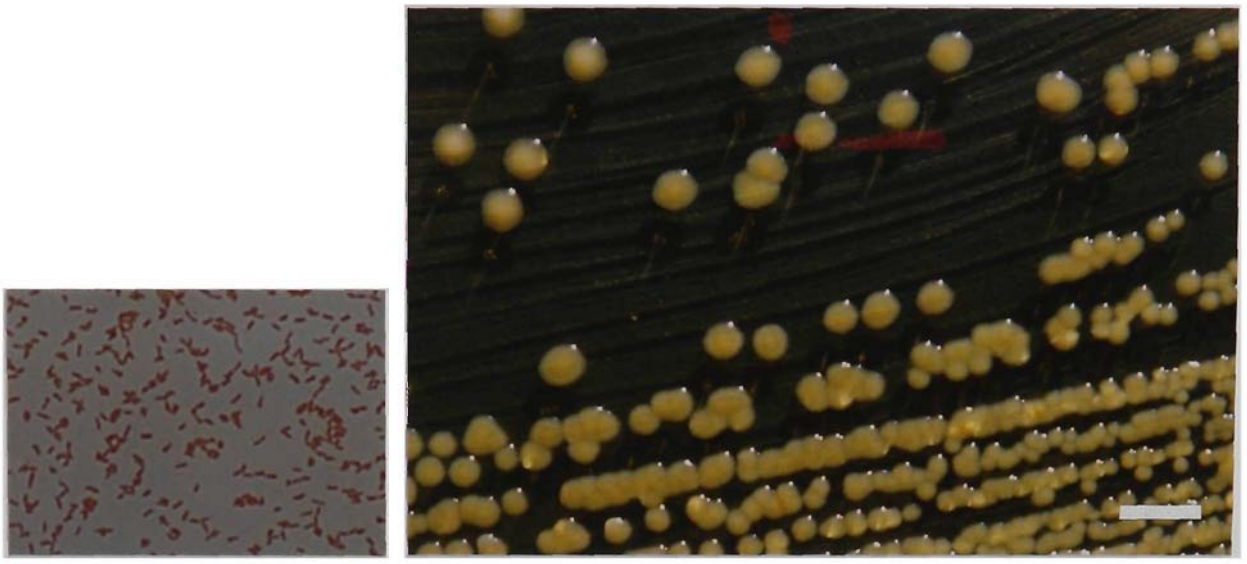
Two types of colony morphology developed when the presumptive greening-associated isolates were grown on complete MIG plates incubated at 25°C.

SA01, SA03, SA05, SA06, SA11, RE01, TA01, TA02 and US04 were all circular, convex, with an entire margin, 1 - 4mm in diameter. When first isolated, the

isolates grew as small translucent creamy/yellow pin-point colonies. Upon subculturing these became larger and more pigmented. A creamy/yellow pigmentation was characteristic for these isolates (Figure 2b) although the intensity of the yellow pigment varied from a deep yellow to a paler hue (Figure 3b). Occasionally the colonies appeared creamy/white (Figure 4b) with only a hint of yellow. When the isolates were subcultured at 25°C, small pin point colonies appeared within 2 days and attained a full size and colouration after 2 - 3 days.

Colonies of isolates SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 were flat, circular (occasionally irregular), with an entire margin and 1 - 4mm in diameter. Initially very small (< 1mm in diameter), the colonies of these isolates became larger with subculturing. Pigmentation in this group was not as marked. Colonies either appeared granular with a creamy/brown tinge (Figure 5b) or remained translucent with only a hint of the creamy/brown pigmentation (Figure 6b). At 25°C, these colonies developed slowly taking up to 5 days for pin point colonies to become apparent. Fully pigmented colonies, took up to 10 days to develop.

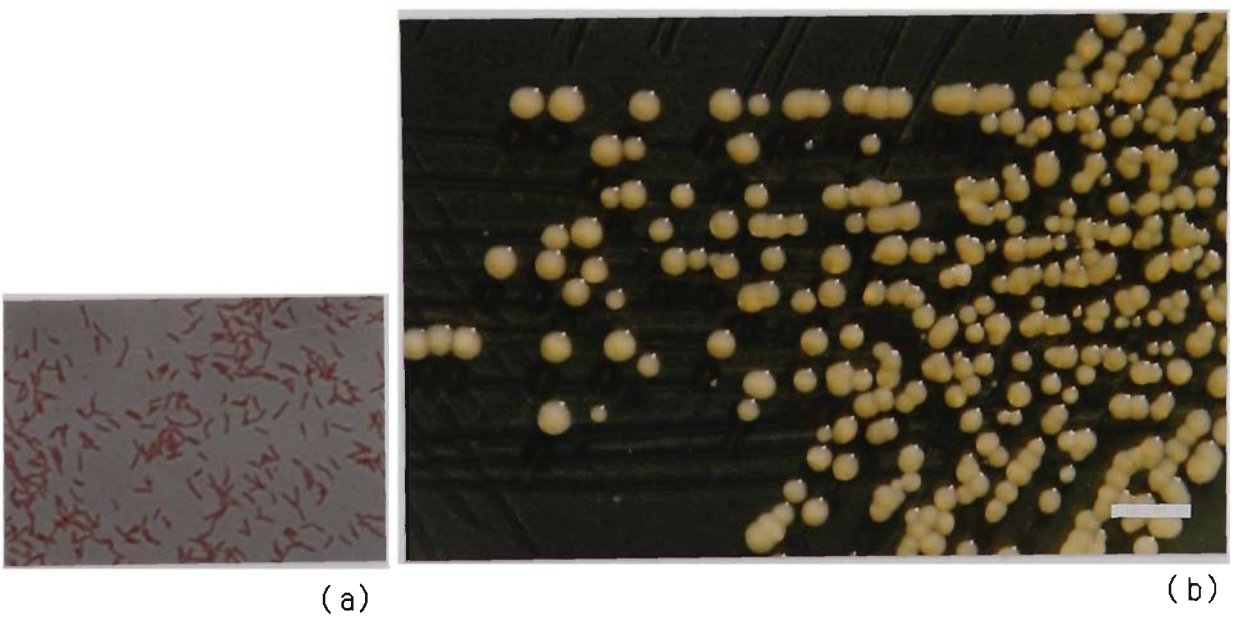
While subculturing SA03, characteristically a deep yellow culture, isolated colonies occasionally exhibited a creamy/white colouration (Figure 8). When sub-cultured, this whitish pigmentation was maintained and did not revert to the deep yellow colouration of the initial isolate. Consequently two variations of isolate SA03 were obtained and retained in pure form: a white variant designated SA03a (Figure 9a) and a yellow variant, SA03b (Figure 10a). The cells of both the white and yellow colony types were identical to one another and to the parent culture, that is, Gram-negative, small, thin, irregular, rods (Figures 9b and 10b).



(a) (b)

FIGURE 2 - SA01 colony morphology and Gram stain

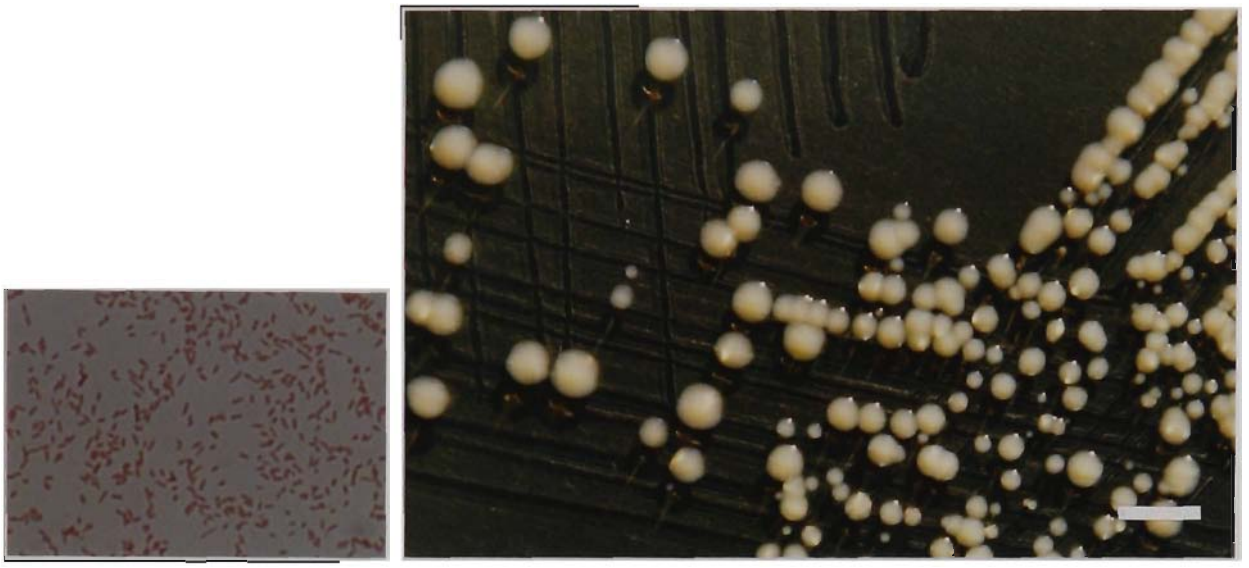
Cells in SA01 colonies grown at 25°C are Gram-negative, small, thin, irregular rods (a). (Magnification: 2000x). SA01 colonies (3 days old and grown at 25°C) are circular, convex, with an entire margin and a creamy/yellow pigmentation (b). (Bar represents 5mm).



(a) (b)

FIGURE 3 - RE01 colony morphology and Gram stain

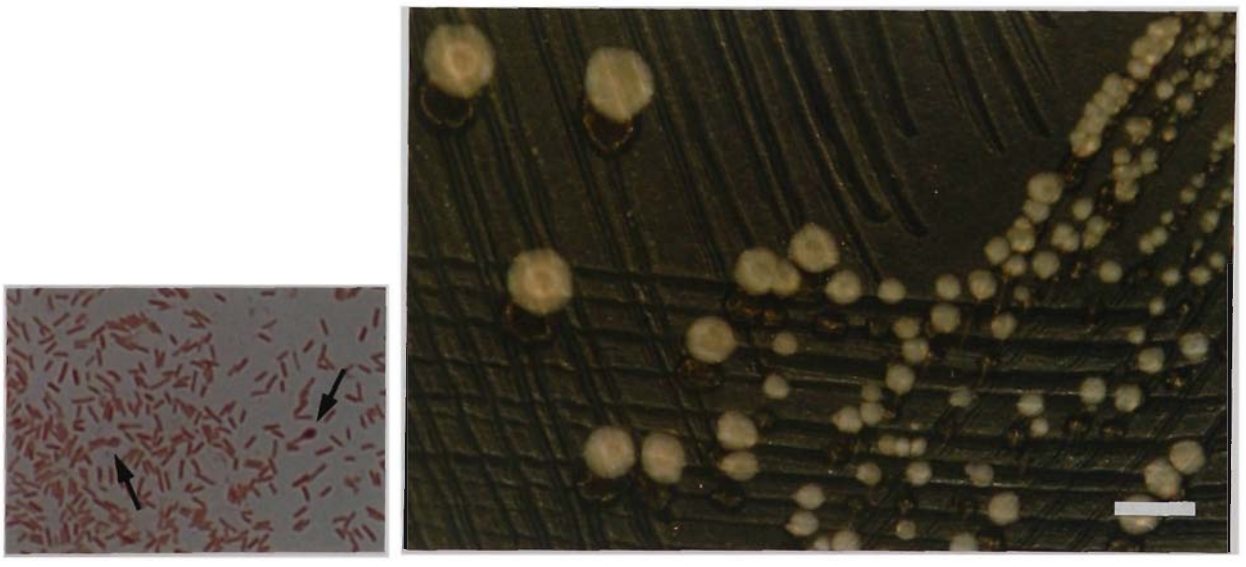
Cells in RE01 colonies grown at 25°C are Gram-negative, thin, irregular rods (a). (Magnification: 2000x). The morphology and pigmentation for a 3 day old RE01 colony grown at 25°C is illustrated in (b). The colonies are paler yellow than those of SA01. (Bar represents 5mm).



(a) (b)

FIGURE 4 - SA11 colony morphology and Gram stain

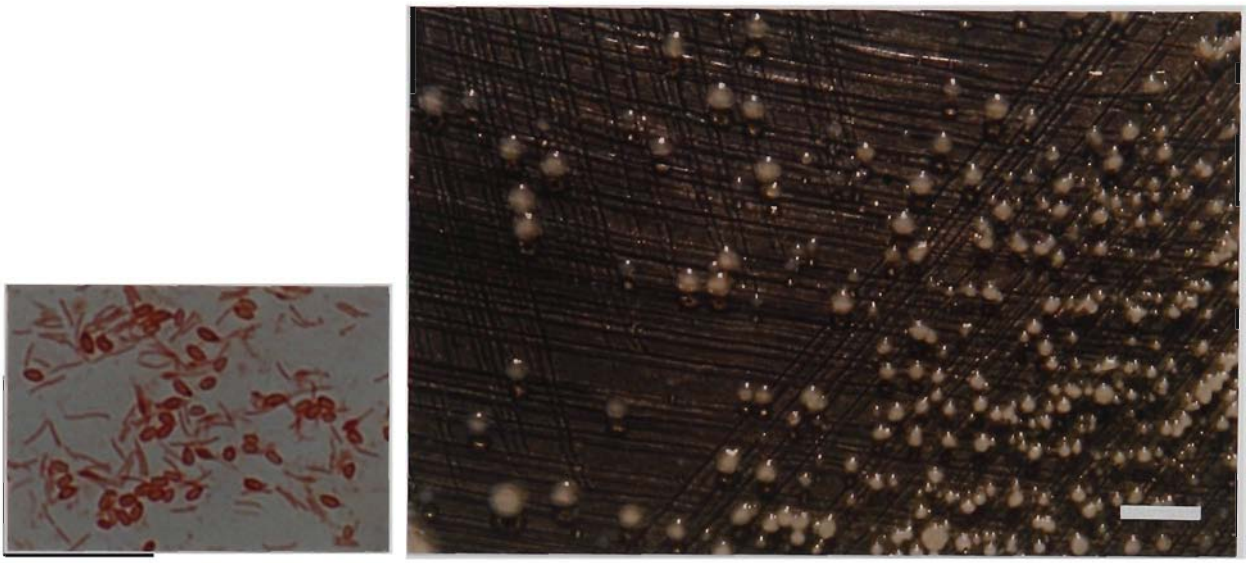
The cells in SA11 colonies grown at 25°C are Gram-negative, small, short, irregular rods (a). (Magnification: 2000x). SA11 colonies (3 days old and grown at 25°C) are circular, convex, with an entire margin and appear almost white with only a hint of yellow (b). (Bar represents 5mm).



(a) (b)

FIGURE 5 - SA09 colony morphology and Gram stain

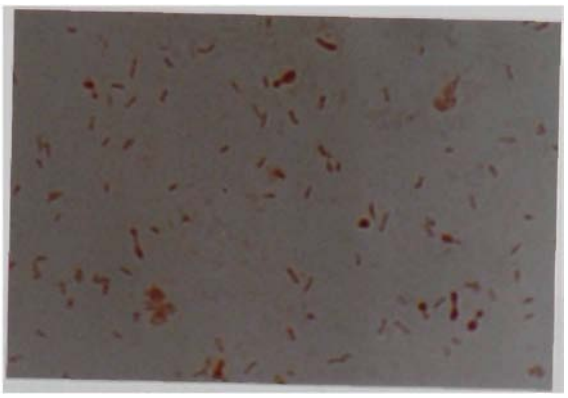
The cells in SA09 colonies (5 - 6 day old and grown at 25°C) are Gram-negative rods with little pleomorphism (a). Sub terminal spore-like structures are seen (arrowed). (Magnification: 2000x). The colonies are flat and occasionally irregular with a transluscent brownish tinge (b). (Bar represents 5mm).



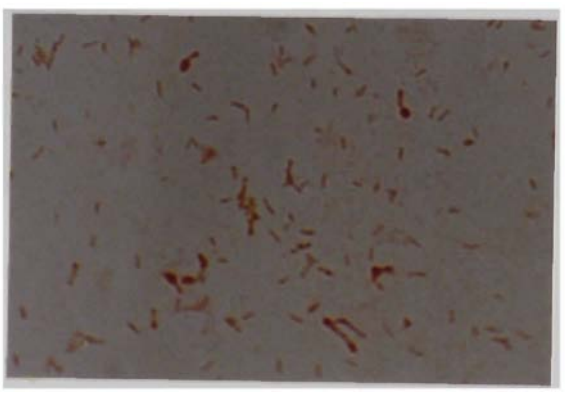
(a) (b)

FIGURE 6 – SA07 colony morphology and Gram stain

Cells in SA07 colonies (7 days old and grown at 25°C) are Gram-negative, long, thin rods (a). An abundance of subterminal spore-like structures are evident. (Magnification: 2000x). The colonies are characteristically small and show little pigmentation (b). Most appear granular or translucent. (Bar represents 5mm).



(a)



(b)

FIGURE 7 – SA01 Gram stain

Gram stains of cells from a 4 day old plate culture of isolate SA01 illustrate the presence of bulbous structures that develop at the terminal ends of the cells (a) and (b). Similar Gram-negative staining structures also ocurred in stained smears of cells of the other isolates. (Magnification: 2000x).

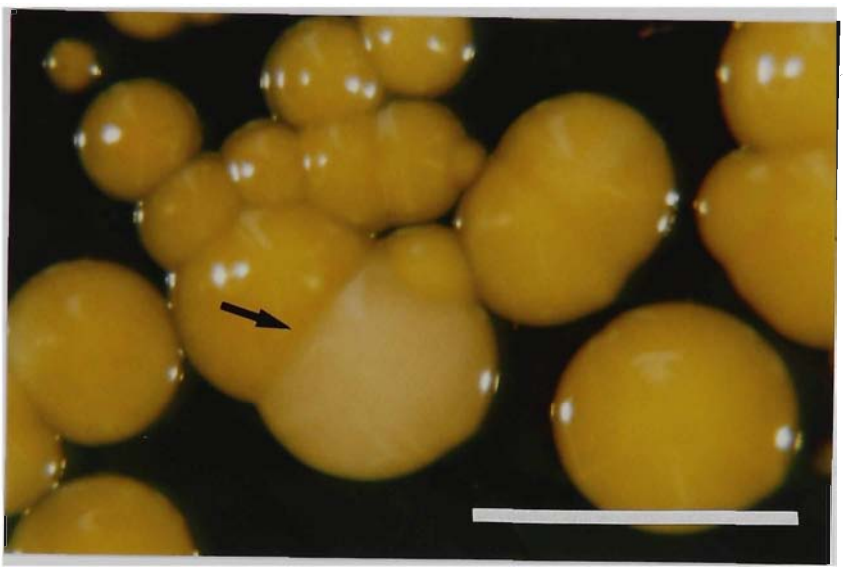
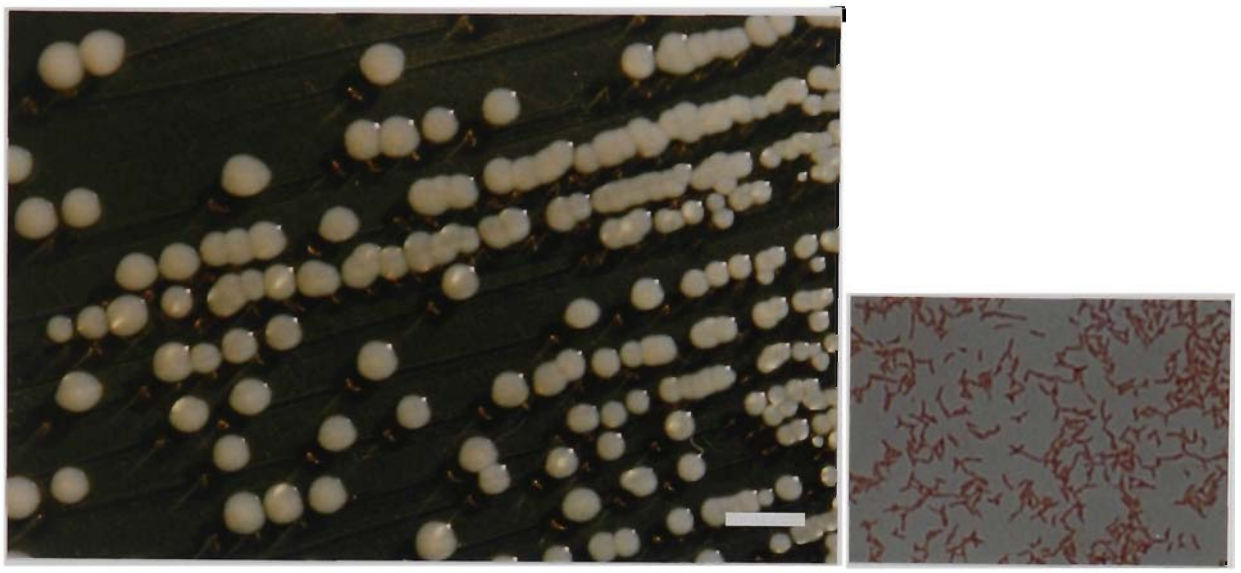


FIGURE 8 – SA03 plate culture

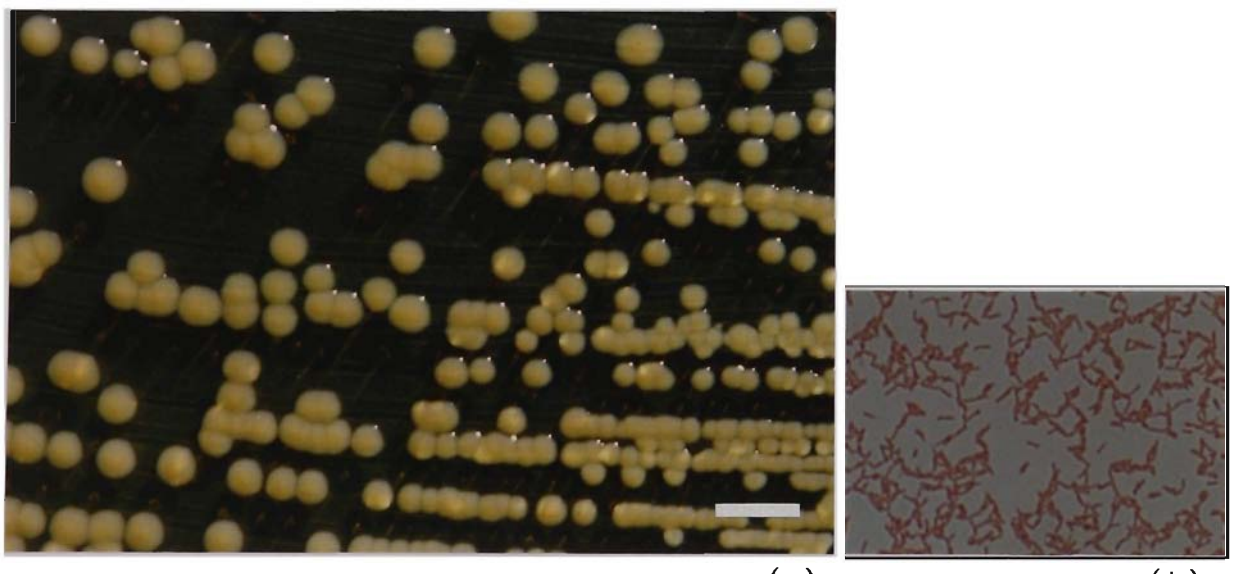
The characteristic deep yellow colony pigmentation for a SA03 plate culture at 25°C is illustrated which after subculturing several times, contained a single creamy/white colony. (Bar represents 5mm).



(a) (b)

FIGURE 9 – SA03a colony morphology and Gram stain

SA03a colonies (3 days old and grown at 25ºC) maintained their creamy/white pigmentation (a) and were morphologically identical to SA03b. (Bar represents 5mm). The cells in these colonies (b) were Gram-negative, thin, irregular rods. (Magnification: 2000x).



(a) (b)

FIGURE 10 – SA03b colony morphology and Gram stain

SA03b colonies (3 days old and grown at 25ºC) illustrating the deep-yellow/creamy-yellow pigmentation (a) characteristic of this isolate. (Bar represents 5mm). The cells in these colonies (b) were Gram-negative, thin, irregular rods. (Magnification: 2000x).

3.1.2.2 Colony ultrastructure

Sections were cut from whole SA01, SA03, SA09, SA10 and TA02 colonies grown on complete MIG medium at 25°C. The bacterial cells in the colony sections were negatively stained and viewed under the TEM. Both young (1 - 2 days) and older (5 days) colonies of the SA01, SA03, SA09 and SA10 isolates were processed as described in section 2.6.1.

Young colonies, 1 - 2 days old, were made up entirely of relatively uniform, thin, rods 1750 - 2000nm long and 250nm wide, randomly distributed throughout the colony. A representative cross section of a SA01 colony is provided (Figure 11). There was little variation from the centre to the perimeter of the colony. In cross sections of older colonies, however, several morphological variations were observed within a single colony. A representative cross section of a SA01 colony is provided (Figure 12). The long thin cells, previously described, appeared wider, measuring 500 x 1500nm and occurred along the surface and perimeter of the colony. Larger round shaped structures occurred towards the centre and older part of the colony. These round structures all appeared granular, were much wider than the long thin cells and showed a markedly varied size ranging in diameter from 375nm to 2500nm (Figure 13). In several cases the round shape appeared to be lost altogether resulting in very large and irregular morphological forms that may have developed from the long thin rods observed in younger colonies.

3.2 Growth curves

Liquid culture growth curves of isolates SA01, SA03, SA05, SA09, SA10, TA01 and TA02 grown at both 25°C and 35°C in complete MIG medium are shown in figures 14, 15, 16, 17, 18, 19 and 20 respectively. Each plot represents the combination of several independent experimental runs carried out under identical conditions. At

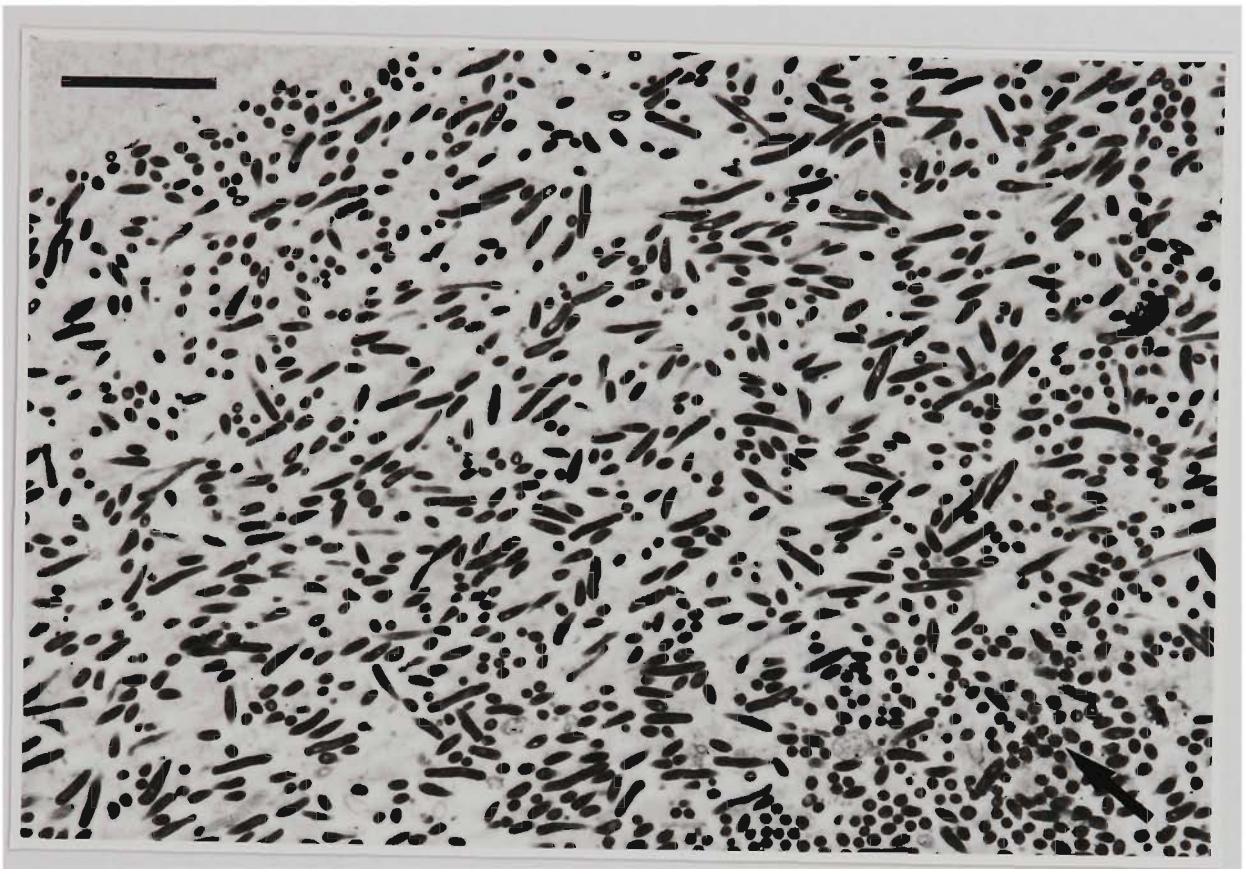


FIGURE 11 – Cross section through a 2 day old SA01 colony

A TEM micrograph illustrating the cell morphology and distribution in a section through a 1 - 2 day old colony grown on complete MIG medium at 25°C. The arrow indicates orientation within the colony from the centre to the surface. (Bar represents 5.0µm).

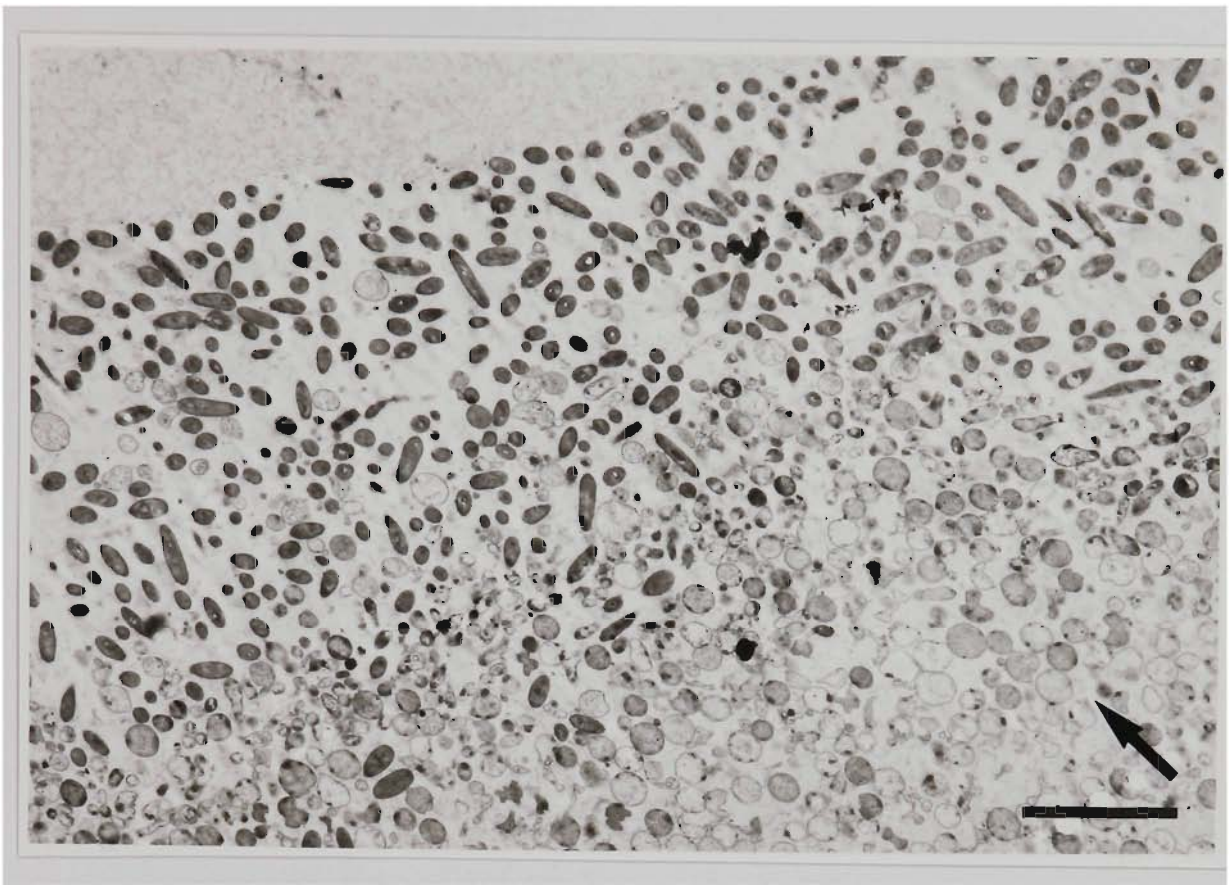
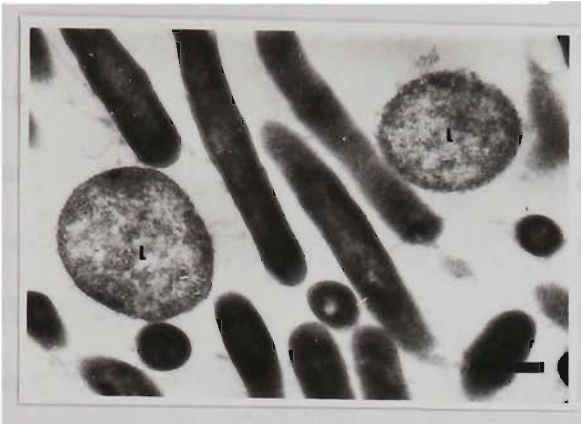


FIGURE 12 - Cross section through a 5 day old SA01 colony

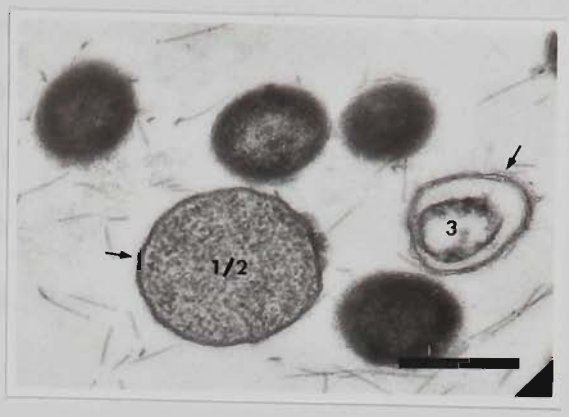
A TEM micrograph illustrating the cell morphology and distribution in a section through a 5 day old colony grown on complete MIG medium at 25ºC. The long thin actively growing cells appear wider than those observed after 2 days of growth and occur along the surface and perimeter while round form structures predominate in the centre of the colony. The arrow indicates orientation within the colony from the centre to the surface. (Bar represents 5.0µm).

FIGURE 13 – "Round forms" observed in 5 day old colony cross sections

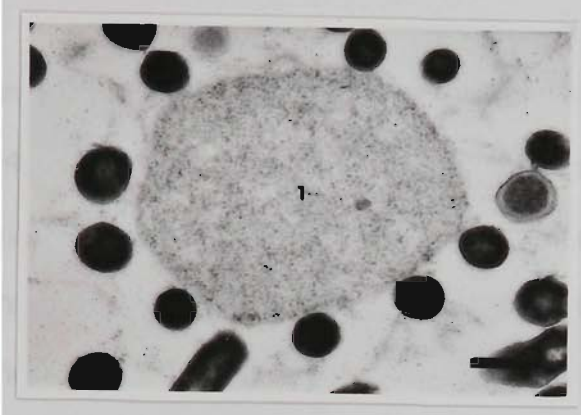
TEM micrographs of the morphological variations in round structures observed in 5 day old colonies. Although most were approximately 2 - 3 x larger in diameter than the rods in (a^1) and (b^1), very large structures were occasionally seen (c^1). Several adopted club-shaped forms (d), (e) and (f). Traces of a distinct cell wall was apparent in (b) and (d) (arrowed). Generally, the round forms contained cellular material which appeared granular (b^2) and (g^2). Empty shells, however, also occurred (b^3), (d^3) and (g^3). (Bar represents 0.5µm).



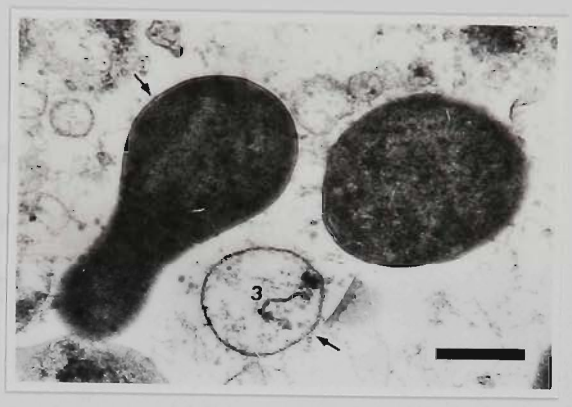
(a)



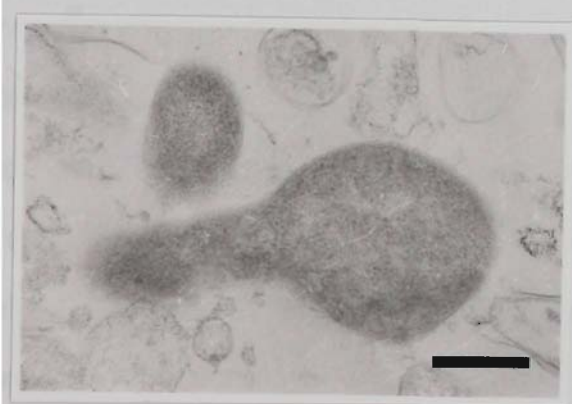
(b)



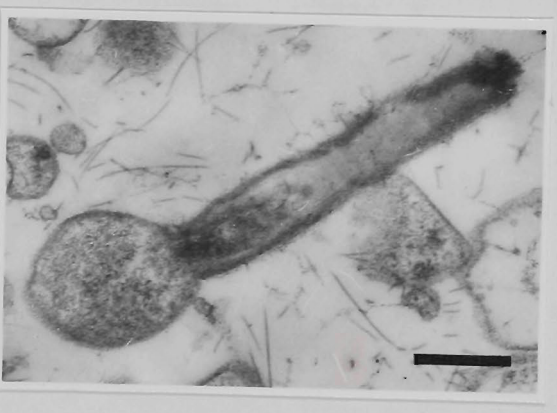
(c)



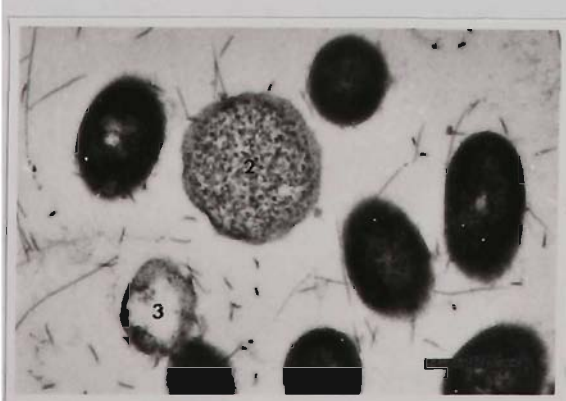
(d)



(e)



(f)



(g)

first the growth curve experiments were carried out directly after the initial transfer of the inoculum from plate cultures stored at 4°C or cultures stored in dH_2O at 10°C to broth. However, in these cases the lag phase of growth was highly variable extending from a few hours to days in length. Consequently, a double stage inoculation procedure was adopted in which the bacteria were transferred to broth, allowed to acclimatize (Section 2.5.1) and subsequently sub-inoculated into the experimental flasks (Section 2.5.2). In this way it was possible to standardize the inoculation procedure and reduce the variability in the lag phase of growth.

Growth curves for isolate SA01 at both 25°C and 35°C were very similar (Figure 14). The lag phase extends from time 0 to approximately 10 hours. This was followed by the exponential (log) phase during the next 80 hours. The stationary phase of growth lasted for approximately 100 hours and was followed by the death phase lasting for approximately 60 hours.

Growth curves for the other isolates were also similar at 25°C and 35°C. A noticeable difference in growth, however, and applicable to all the isolates, is the higher bacterial titre reached during stationary phase at 25°C. Although rates of growth were similar during the exponential phase at the two temperatures, this rate was maintained longer at the lower temperature thus resulting in a greater cell density at 25°C.

The rates of growth for isolates SA01, SA03, SA05, SA10, TA01 and TA02 during the exponential phase at both 25°C and 35°C were not significantly different with a 95% confidence level (Appendix VIII). Only isolate SA09 had a significantly higher rate of growth at 35°C than at 25°C.

Based on analyses of the stationary phase y-intercepts, there was a significant difference between the two temperatures for growth of isolates SA01 and SA05 at

the 0.1% level suggesting that the bacterial titre attained at 25ºC is higher than at 35ºC as determined by the absorbance readings. Such observations requiring horizontal fits for the stationary phase data could not be made for the SA03, SA09, SA10, TA01 and TA02 cultures.

From observations of negatively stained samples viewed with the TEM, the isolates studied followed two distinct patterns of morphological changes with growth. Isolates SA01, SA03, SA05, TA01 and TA02 differed from SA09 and SA10.

The lag phase for SA01, SA03, SA05, TA01 and TA02 (Figure 21a) consisted predominantly of long thin rods that appeared to be slightly irregular, measuring 2,088 ± 585nm (n = 50) long and 200nm wide. With the onset of growth at 25ºC, early log phase, these cells were short and fat (Figure 21b), measuring 1,152 ± 298nm (n = 50) long and 200 - 400nm wide. During the log phase the cells became more irregular and distinctly pleomorphic (Figure 21c). During the stationary phase of growth these cells maintained the pleomorphic appearance although they became short and small round structures began to appear (Figure 21d). Lysing cells also became evident, which increased in number as the stationary phase proceeded (Figure 21e). In the death phase, the culture comprised of cells in various stages of lysis, with ghosts and an abundance of circular cell-like structures believed to be the "round form" of the organism (Figure 21f).

Morphological variations with growth at 35ºC followed a pattern very similar to that at 25ºC during the late lag/early log phases of growth. The long thin cells in the lag phase became shorter and fatter with growth and more irregular during the log phase. However, as the culture at 35ºC reached the late log/early stationary phases, the cells did not shorten as at 25ºC. Instead, these developed bulbous areas giving club-shaped or dumb-bell shaped appearances (Figure 22a). The cells were 2,048 ± 370nm (n = 50) long and 200 - 600nm wide depending on

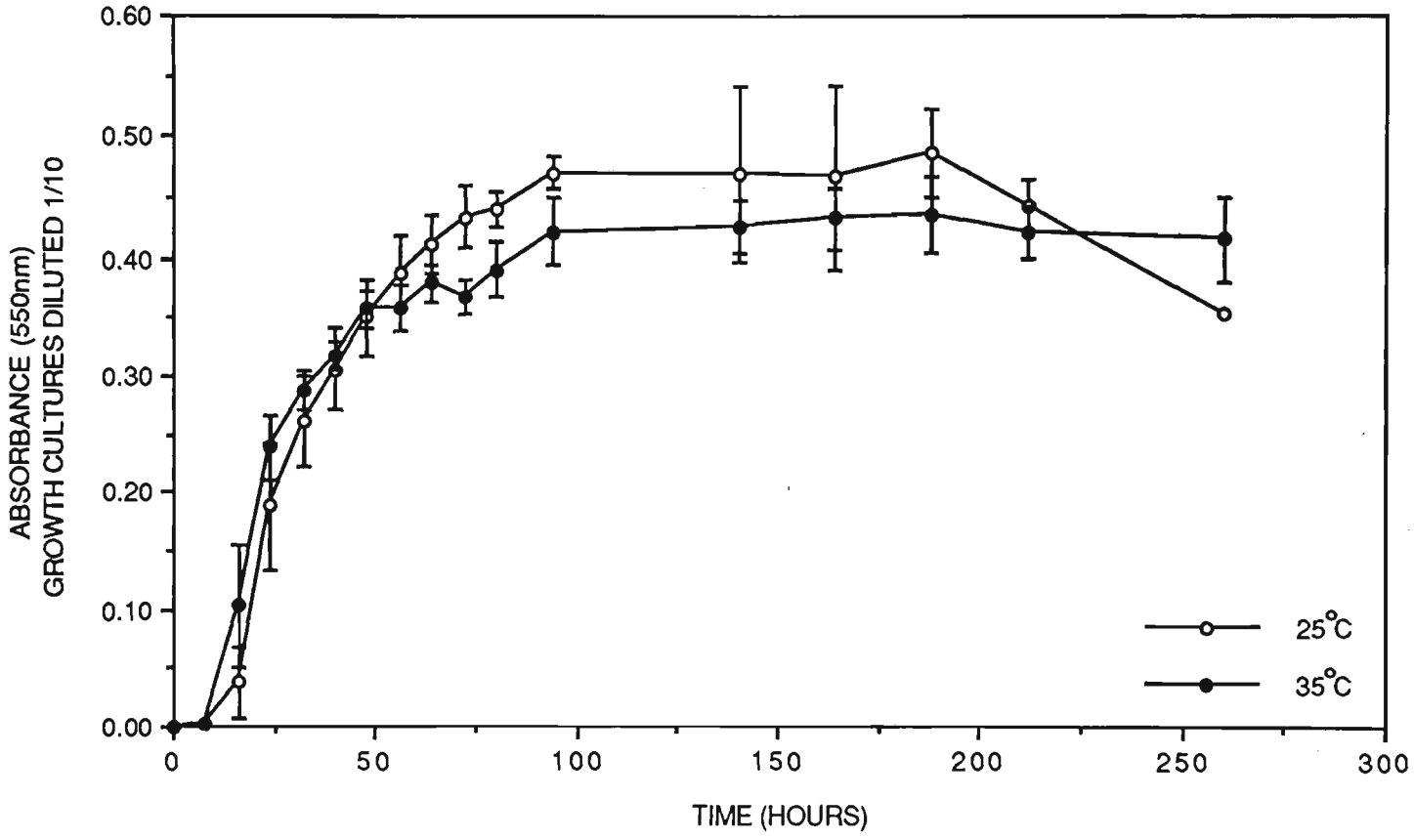


FIGURE 14 - Growth curves for isolate SA01 at 25°C and 35°C

The absorbances of samples diluted 1/10 read at 550nm in a 1cm light path were plotted as a function of time. Bars represent the standard deviations of the means calculated from 6 replicate curves.

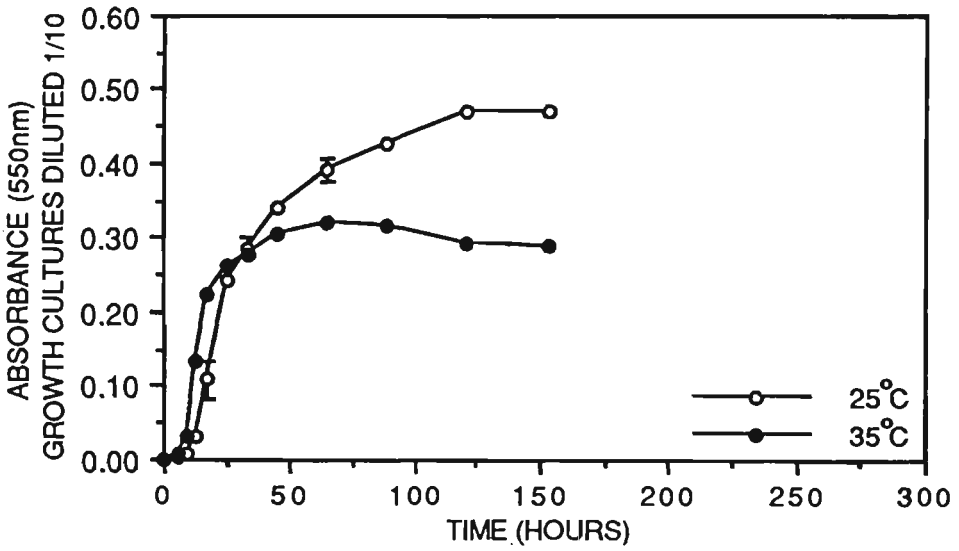


FIGURE 15 - Growth curves for isolate SA03 at 25°C and 35°C

Bars represent the standard deviations of the means calculated from 2 replicate curves.

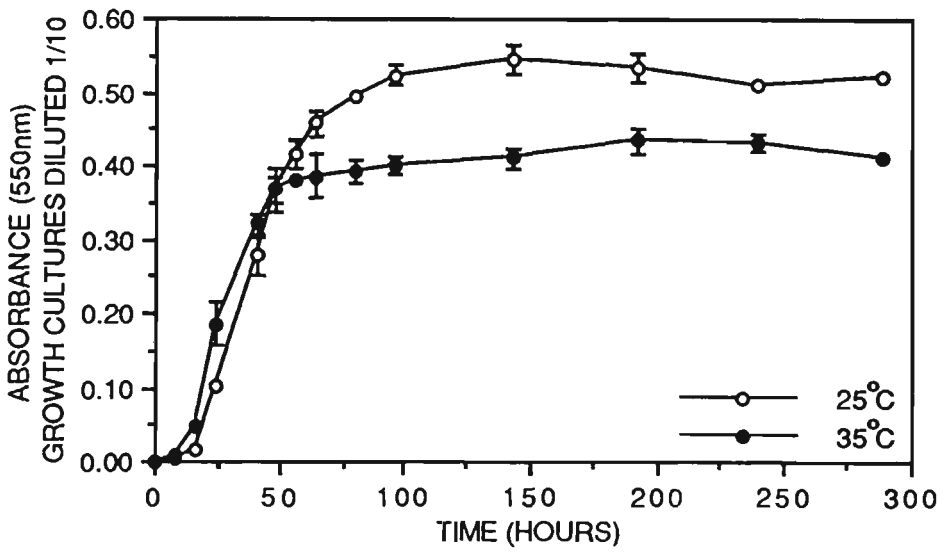


FIGURE 16 - Growth curves for isolate SA05 at 25°C and 35°C

Bars represent the standard deviations of the means calculated from 3 replicate curves.

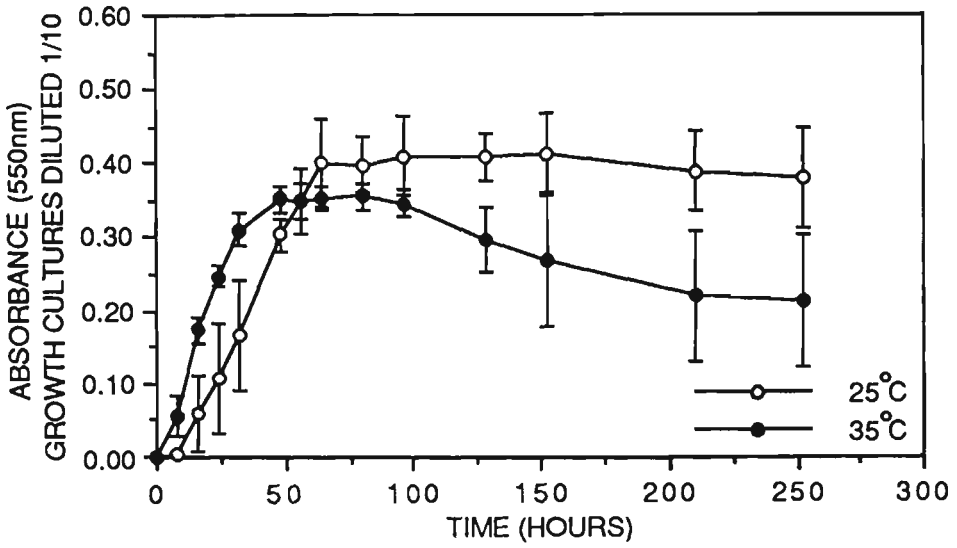


FIGURE 17 - Growth curves for isolate SA09 at 25°C and 35°C

Bars represent the standard deviations of the means calculated from 6 and 5 replicate curves at 25°C and 35°C respectively.

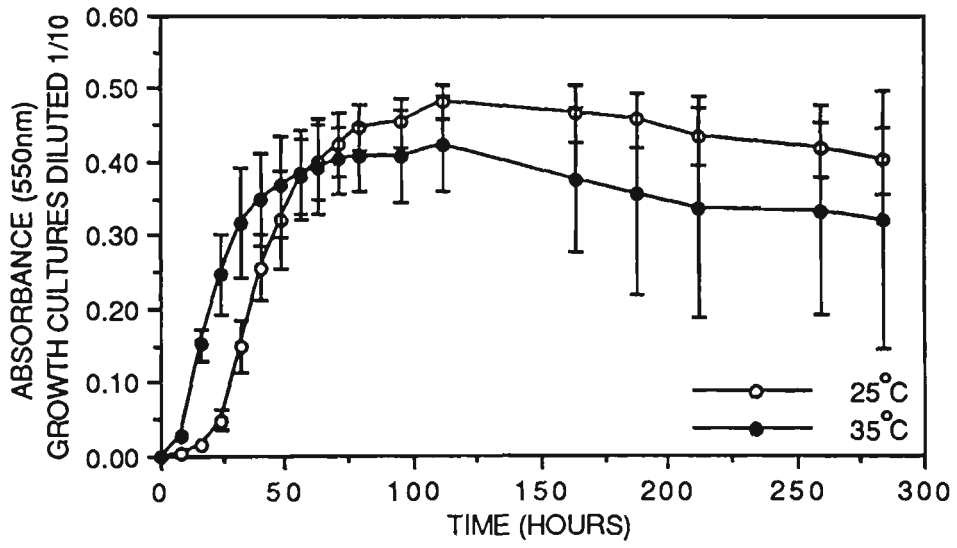


FIGURE 18 - Growth curves for isolate SA10 at25°C and 35°C

Bars represent the standard deviations of the means calculated from 6 replicate curves.

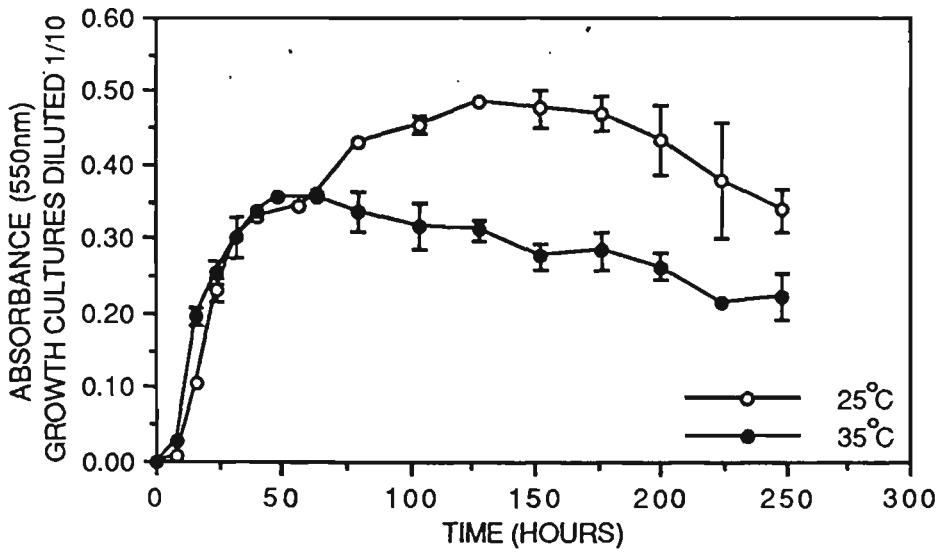


FIGURE 19 - Growth curves for isolate TA01 at 25°C and 35°C

Bars represent the standard deviations of the means calculated from 3 replicate curves.

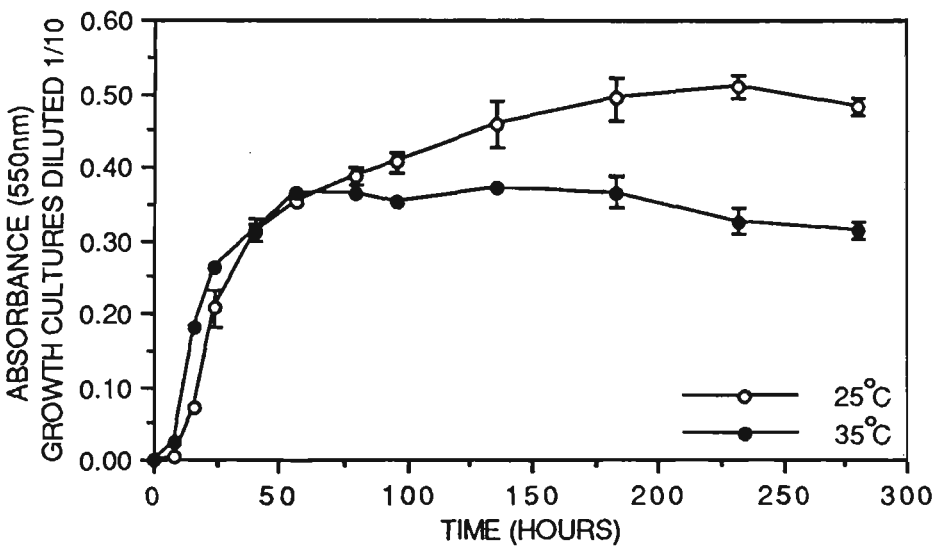


FIGURE 20 - Growth curves for isolate TA02 at 25°C and 35°C

Bars represent the standard deviations of the means calculated from 3 replicate curves.

the extent of bulging. These bulbous shapes were maintained throughout the stationary phase during which the lysing cells appeared highly irregular (Figure 22b). Once again, as at 25°C, the death phase was marked by cells in various stages of lysis, ghosts and the circular cell-like or "round form" structures.

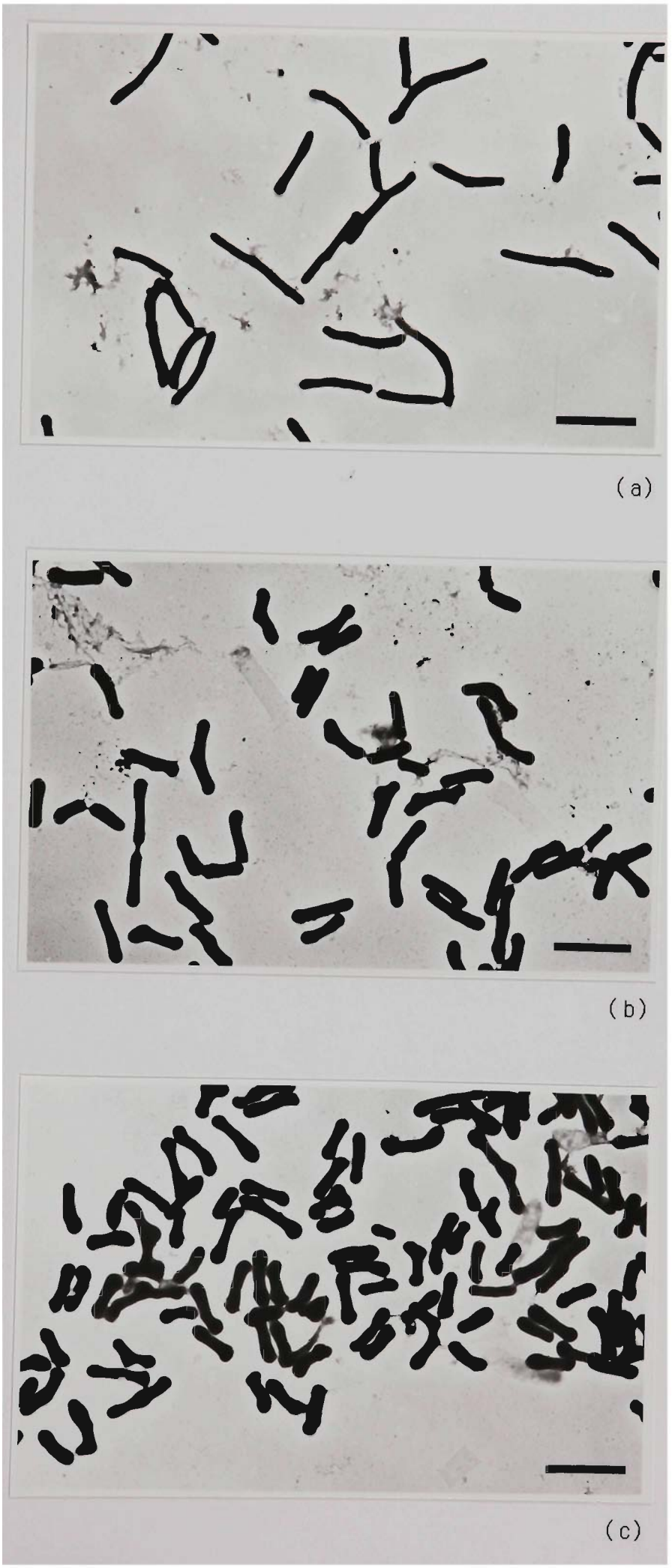
The morphological variations with growth for isolates SA09 and SA10 were totally different from that described for the other isolates. The long thin cells of the lag phase (Figure 23a) measuring 1,832 ± 363nm (n = 50) long and 200 - 300nm wide became short and slightly thick in the early log phase of growth (Figure 23b). During the late log phase (Figure 23c) and proceeding into the stationary phase of growth, these cells became even fatter and less electron dense (Figure 23d). These cells measured 1,608 ± 355nm (n = 50) long and 500nm wide. Throughout the stationary (Figure 23e) and death phases (Figure 23f), the cells appeared to be lysing, resulting in a culture that consisted of lysed cells, ghosts and cellular debris. Once again, circular cell-like structures similar to the "round form" of the putative greening-associated bacterium were evident. This sequence of morphological variation with growth occurred at both the 25°C and 35°C temperatures.

During growth at both 25°C and 35°C, "round form" structures occurred towards the end of the growth cycle for isolates SA01, SA03, SA05, SA09, SA10, TA01 and TA02 (Figure 24). These "round form" structures varied greatly in size, with a diameter of 728 ± 251nm (n = 50) and appeared to originate from the bulbous thickenings that developed on the rods earlier in growth cycle. With the development of the "round form" an apparent associated loss in cell wall rigidity and structure of the rod occurred.

A typical example of the ghost cell morphology for isolates SA01, SA03, SA05, SA09, SA10, TA01 and TA02 stationary and death phase cultures at both temperatures is illustrated (Figure 25).

FIGURE 21 - TEM micrographs of the morphological changes in isolate SA01 grown at 25°C

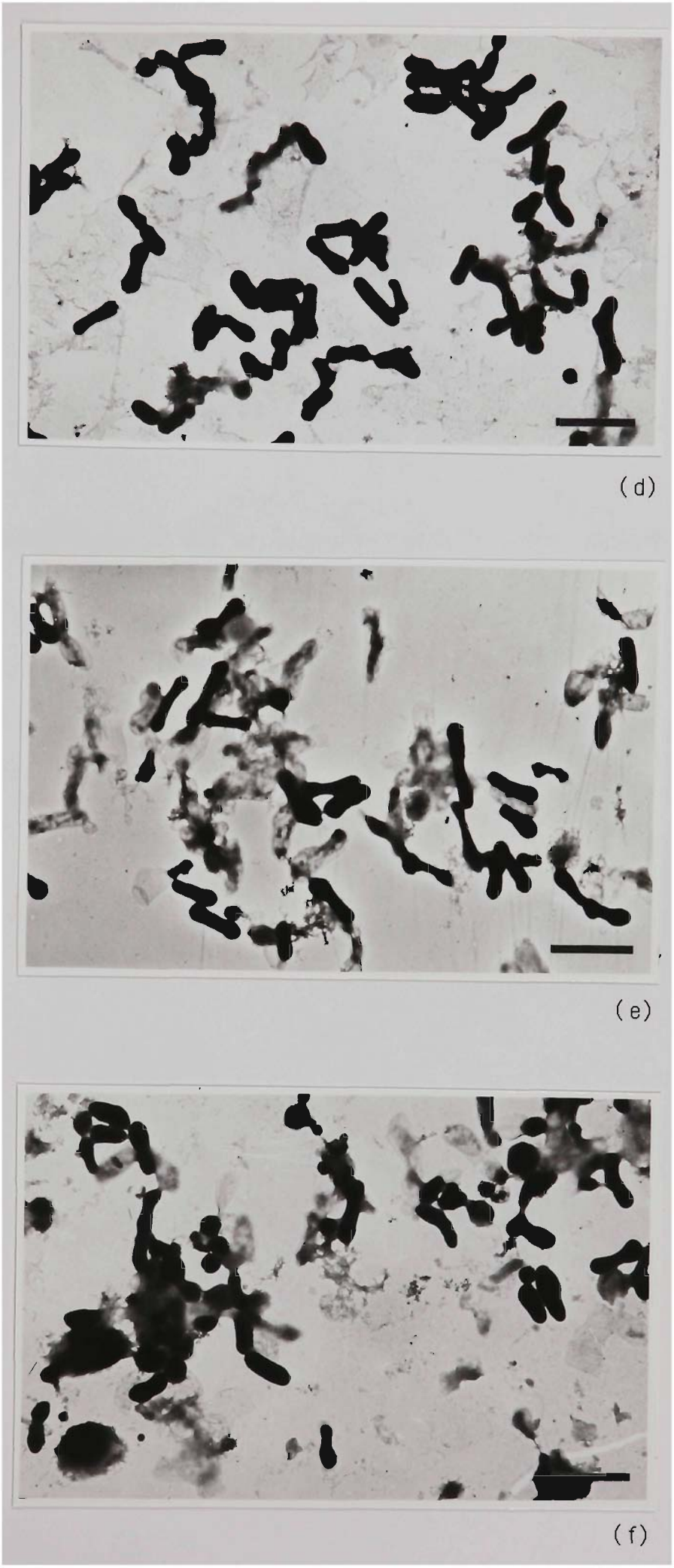
The cell morphology is represented for the lag phase (a), accelerating phase (b), log phase (c), decelerating phase (d), stationary phase (e) and death phase (f) of isolate SA01 grown in complete liquid MIG at 25°C. Samples were negatively stained and viewed with a JEM-100s transmission microscope. (Bar represents 2.0µm). Similar morphological changes with growth occurred for isolates SA03, SA05, TA01 and TA02.



(a)

(b)

(c)



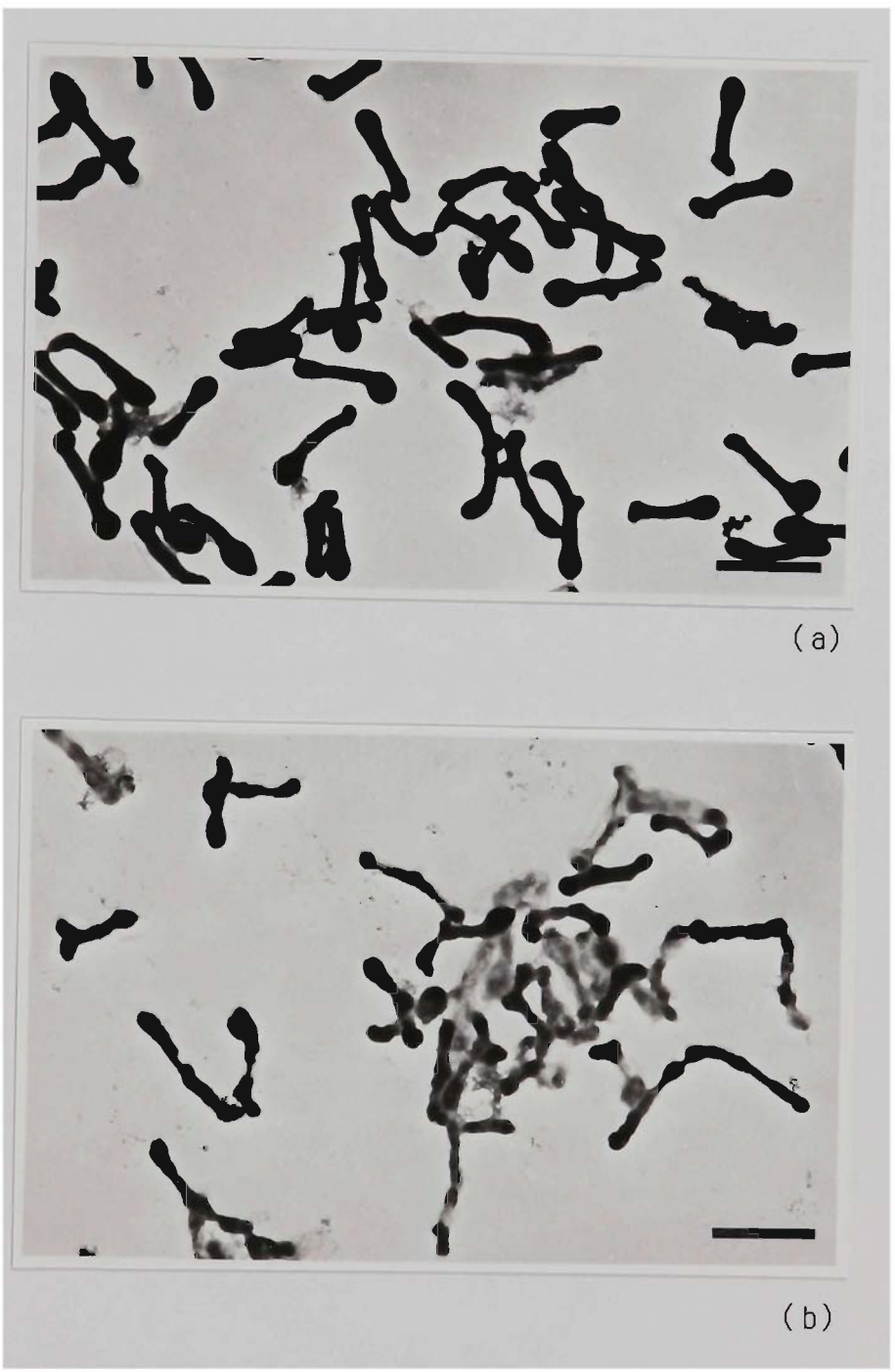
(d)

(e)

(f)

FIGURE 22 - TEM micrographs of the morphological changes in isolate SA01 grown at 35°C

The cell morphology in negatively stained samples from the decelerating phase (a) and stationary phase (b) of growth and viewed with the TEM, are illustrated. (Bar represents 2.0μm). Cell morphologies of isolates SA03, SA05, TA01 and TA02 were the same as SA01 in these phases.

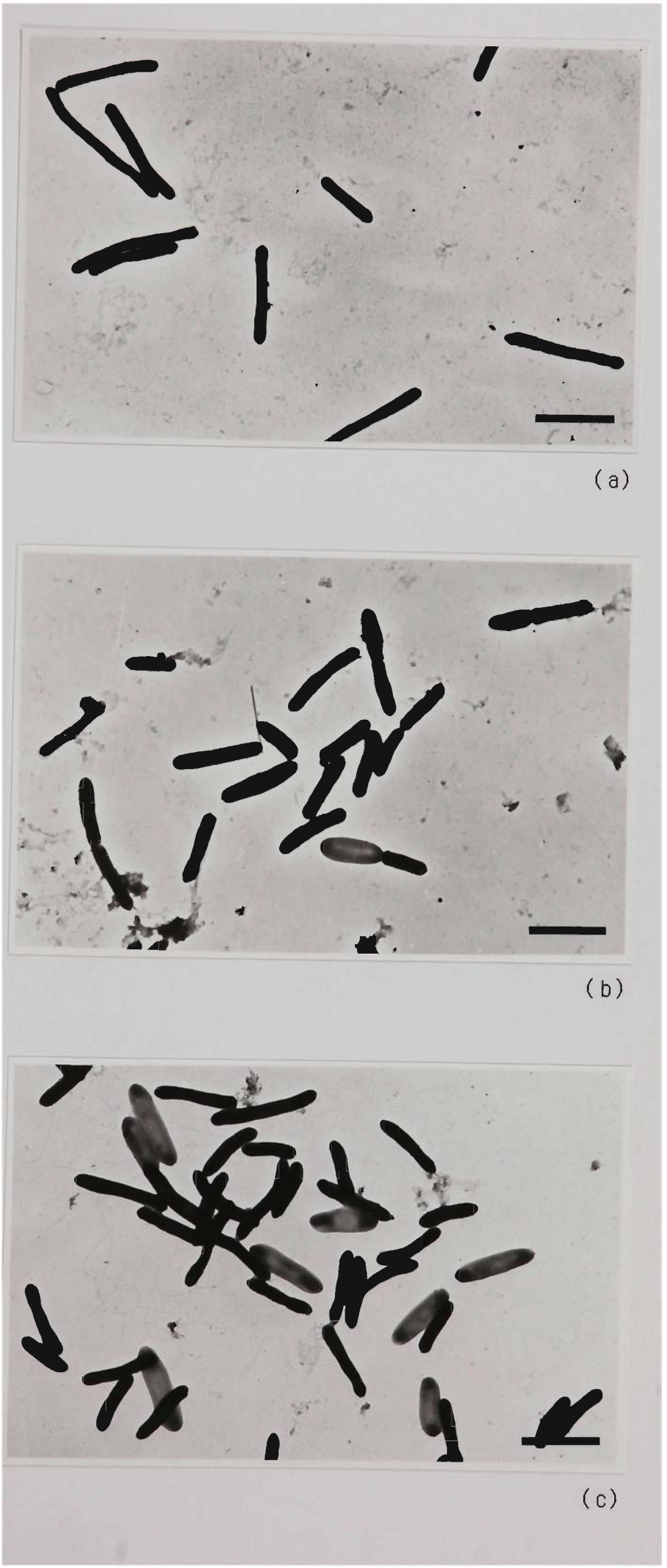


(a)

(b)

FIGURE 23 - TEM micrographs of the morphological changes in isolate SA09 grown at 25°C and 35°C

The cell morphology is represented in TEM micrographs for the lag phase (a), accelerating phase (b), log phase (c), decelerating phase (d), stationary phase (e) and death phase (f) of isolate SA09 grown at 25°C. Samples were negatively stained and viewed with a JEM-100s electron microscope. (Bar represents 2.0µm). The morphological changes for isolate SA09 were the same at 35°C. Similar morphological changes with growth occurred for isolate SA10 at both temperatures.



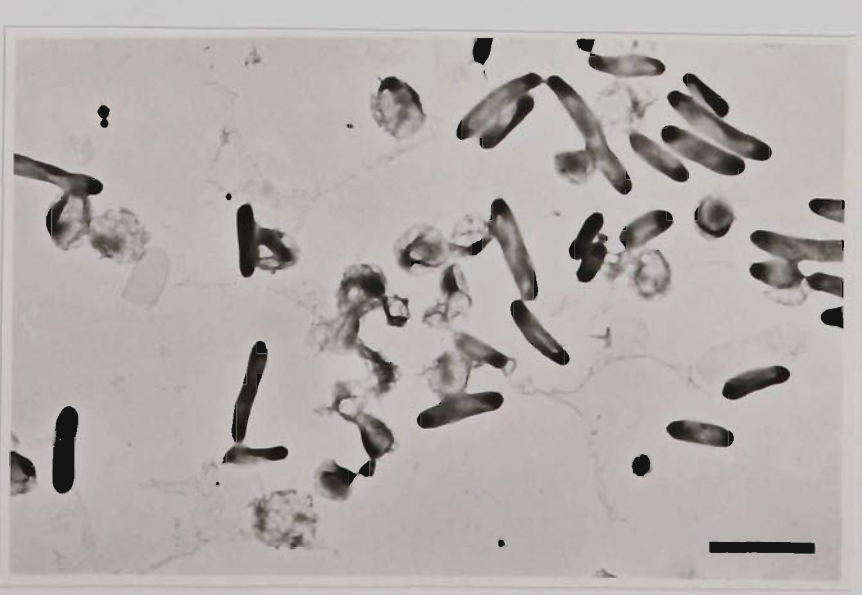
(a)

(b)

(c)

(d)

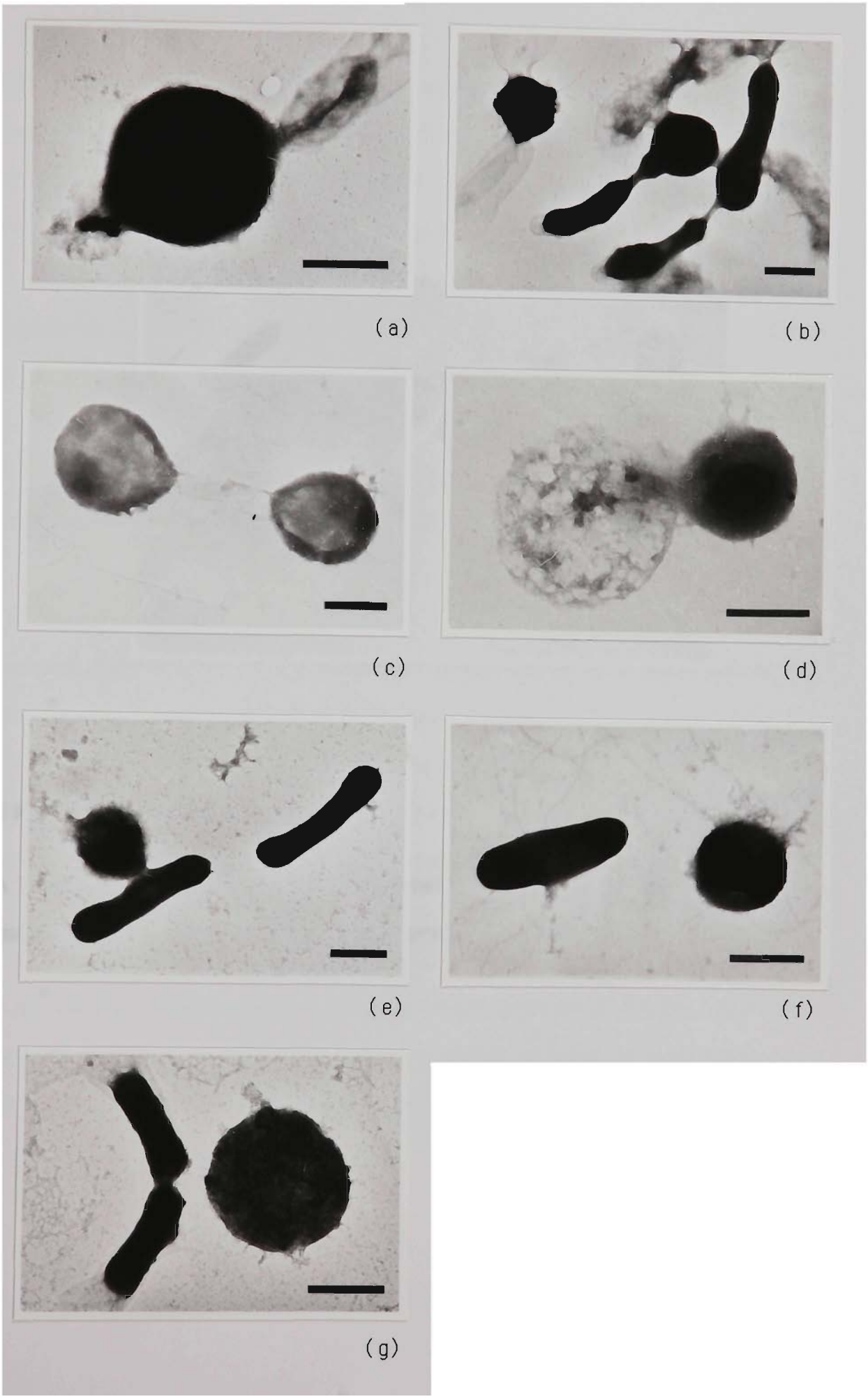
(e)



(f)

FIGURE 24 – "Round forms" in old liquid cultures at 25°C and 35°C

TEM micrographs of negatively stained stationary and death phase cultures illustrate the diversity of the round form morphologies. Figures (a), (b) and (c) suggest that the round form develops from bulbous areas originating centrally and at the terminal ends of the cells. A sheath appears to be associated with the round form in (d). The sizes varied from 1.5 x the diameter (e) and (f), to 5 x the diameter of the rod (g). (Bar represents 0.5µm).


(a)
(b)
(c)
(d)
(e)
(f)
(g)

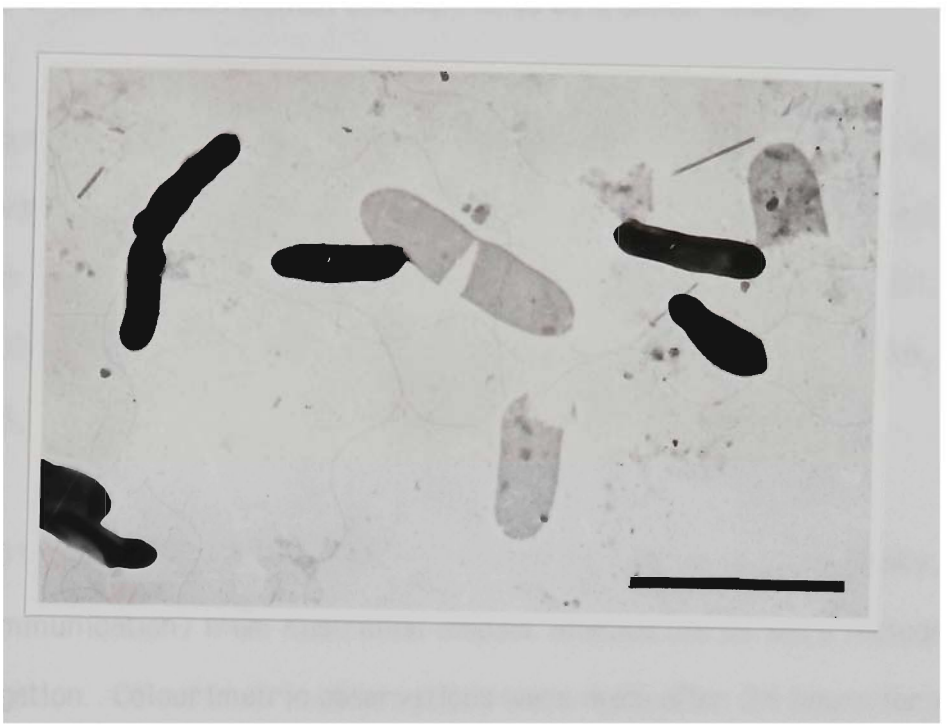


FIGURE 25 - A typical ghost cell

A TEM micrograph of an empty bacterial shell or "ghost cell" observed during the death phase of a culture at 25ºC. (Bar represents 2.0µm).

3.3 Metabolic characterization

Metabolic characterization of the putative greening-associated bacterial isolates was carried out using the Biolog GN microplate (2.4) that differentiates species of Gram-negative bacteria. The utilization of 95 different carbon sources was evaluated by a redox based reaction and monitored by a colour change.

The patterns representing the ability of the isolates to use different carbon sources are collectively illustrated in table 2. Colourimetric observations were made after 24 hours for isolates SA01, SA03a, SA03b, SA05, SA06, SA11, RE01, TA01, TA02, and US04. No colour reactions were obtained for isolates SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 after incubation for 24 hours.

Isolates AU01, AU02 and AU03 (Ref 3.4) and AU04 and AU05 (R.H. Brlansky, personal communication) from Australian dieback affected citrus were included in this investigation. Colourimetric observations were made after 24 hours for AU01, AU02, AU04 and AU05. No colour reactions were observed for AU03. The carbon utilization pattern for AU01 was similar to that of the SA01, SA03a, SA03b, SA05, SA06, RE01, TA01, TA02, and US04 isolates.

Different carbon utilization patterns were observed for isolates SA11 and AU02. AU04 and AU05 appeared to have patterns similar to the SA01, SA03a, SA03b, SA05, SA06, RE01, TA01, TA02, and US04 isolates but were not investigated further as the metabolic tests on AU04 and AU05 were performed at the end of the study.

A more detailed presentation of the identity of the carbon sources and the utilization ability of the isolates is presented in appendix VII.

From Table 2 it becomes evident that many of the cultures share similar carbon utilization patterns suggesting possible metabolic relationships. In an attempt to identify the isolates, several representative patterns, closely resembling that indicated by the shaded and solid spots in table 2, were screened using the Biolog database of reference patterns. The SA01, SA03a, SA03b, SA05, RE01, AU01 and US04 isolates were found to have a 62.6% - 88.8% homology to Clavibacter michiganense subsp. michiganense. In addition a 36.6% homology with Clavibacter michiganense subsp. insidiosum was found for AU01.

3.4 Australian citrus dieback

3.4.1 Symptomology

Symptoms of ACD affected citrus tissue were similar to those documented for greening (Fraser et al., 1966; McClean and Oberholzer, 1965; McClean and Schwarz, 1970). ACD affected trees showed localised areas of sparsely foliated branches with evidence of multiple budding. The leaves not only had the characteristic vein yellowing but the discolouration also ranged from mild blotchy mottles to more severe chlorotic patterns (Figure 26). In some extreme cases, there were clusters of entirely defoliated branches. The Valencia fruit collected from Dareton were small, green and, contrary to the observations by Broadbent (1977[1]), lopsided. Several of the grapefruit collected in the MIA, although showing little discolouration or lopsidedness, were notably smaller than unaffected fruits. Leaf and tree symptoms were quite marked on many Marsh grapefruit trees in the several orchards sampled at Griffith (MIA) in September 1990. These symptoms were not as evident in late November of the same year (R.H. Brlansky, personal communication).

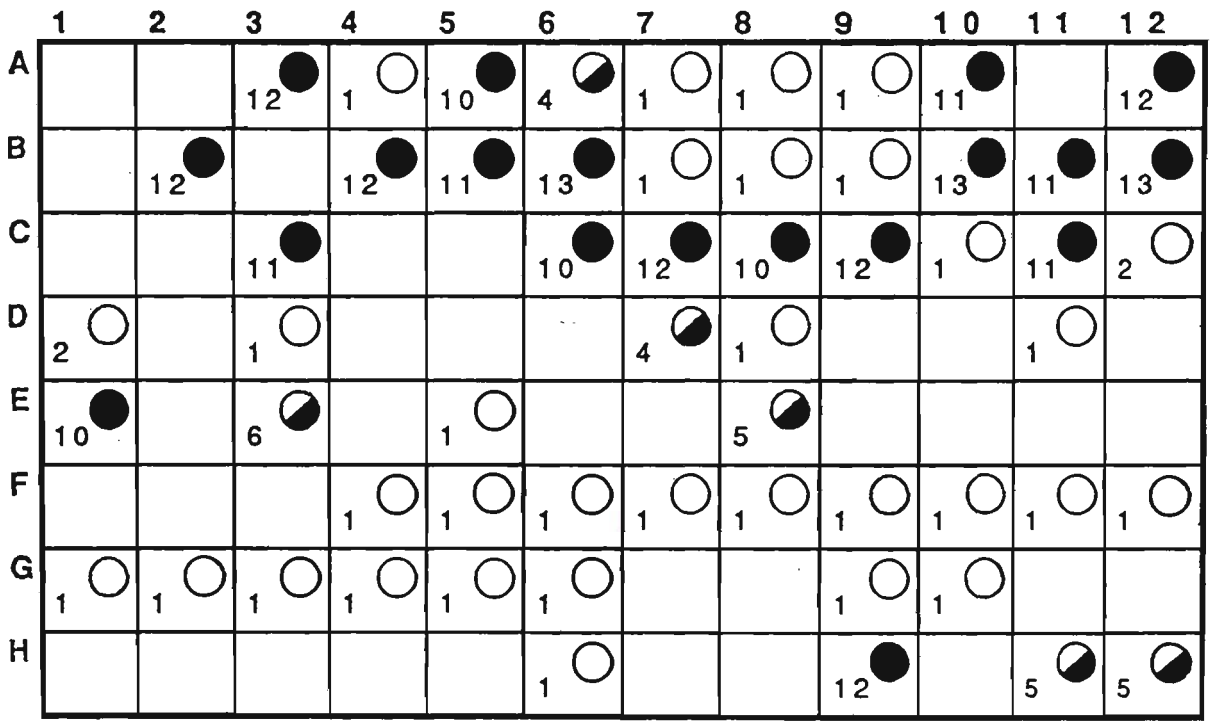


TABLE 2 - Biolog GN carbon utilization patterns

A collective representation of the carbon utilization patterns for isolates SA01, SA03a, SA03b, SA05, SA06, SA11, RE01, TA01, TA02, AU01, AU02, AU04, AU05 and US04 . The numbers in the squares depict the number of cultures out of the 14 tested that were able to use that particular carbon source.

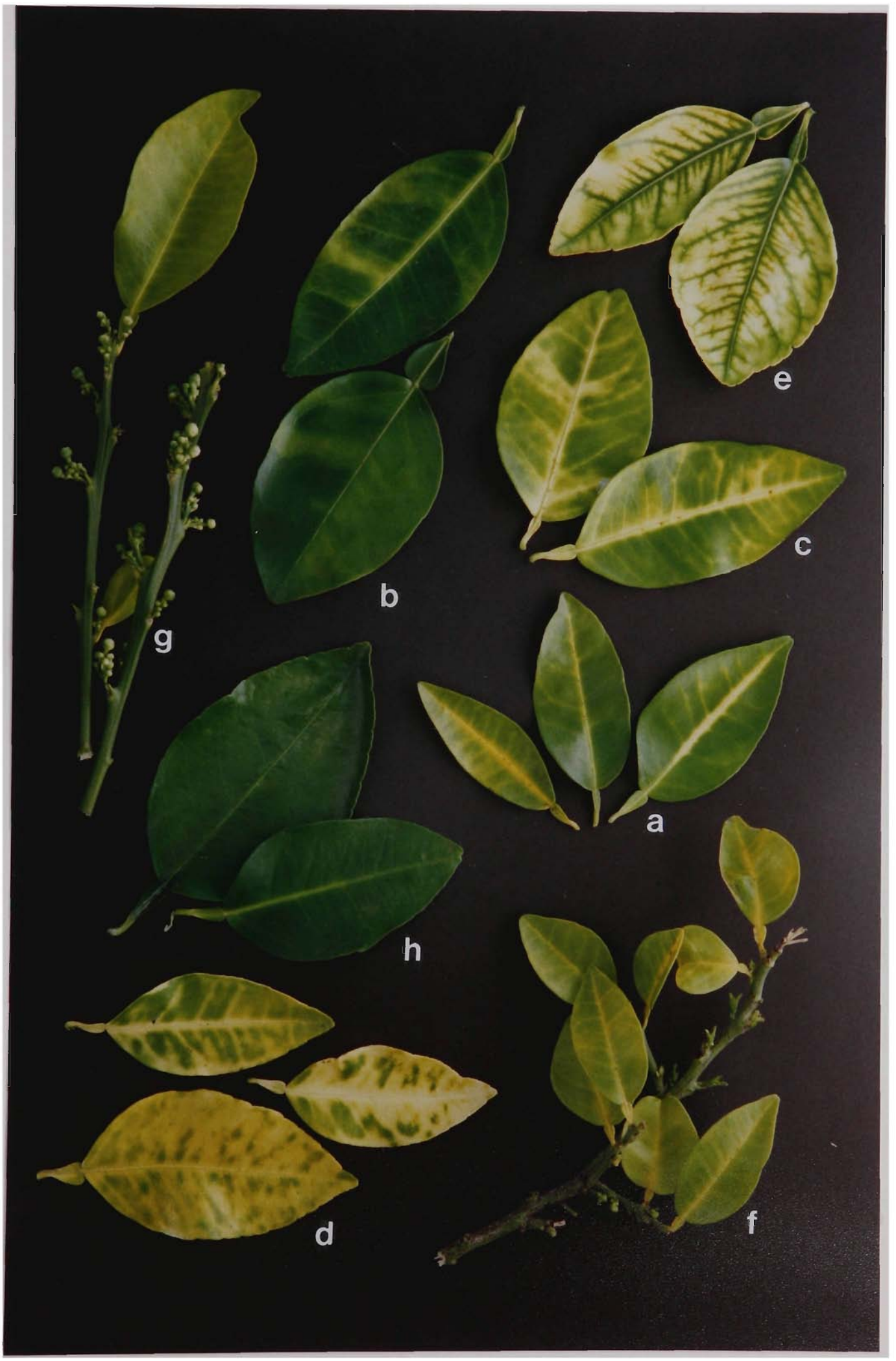
● represents the carbon source utilized by 7 - 14 of the cultures tested,

◪ represents the carbon source utilized by 4 - 7 of the cultures tested and

○ represents the carbon source utilized by 1 - 4 of the cultures tested.

FIGURE 26 - Leaf symptoms from Australian Citrus dieback-affected grapefruit trees

(a) The typical vein yellowing associated with early infection. As this yellowing progresses into the adjacent tissues the leaves exhibit a range of blotchy mottles (b) and (c) or become chlorotic (d) and (e). Leaves may also appear stunted and resemble a zinc deficiency condition (f). Multiple bud development and early flowering is also evident (g). (h) Healthy unaffected control.


g
b
e
c
a
h
d
f

3.4.2 Isolations

Growth occurring within 3 - 4 days usually indicated a bacterial isolate that did not correspond to the morphological description of the greening-associated bacterium. Such presumed non-greening-associated bacteria were common in many isolation attempts and included: Gram-positive short, fat rods; Gram-positive coccobacilli; Gram-positive cocci; Gram-variable large rods and yeasts. Flasks which appeared faintly turbid after 7- 10 days, usually consisted of small thin Gram-negative rods similar to the descriptions of the putative greening-associated bacteria (Section 3.1.1). The ability of these bacteria to pass through a 0.45µm filter was tested as described in section 2.7.3. If growth was not observed after about 20 days, the flasks were discarded.

From a total of 87 independent isolations, 48 were made from the leaf midribs of symptomatic leaves and the remaining 39 from the fruit columella. Nine isolates with some properties similar to those described for isolates from South African, Reunion and Taiwan greening samples were obtained. These isolates were Gram-negative, small, short, irregular rods with thickened ends or Gram-variable (staining both Gram-positive and Gram-negative), small, irregular rods. One of the isolates was a Gram-negative, very thin, filamentous rod, morphologically very similar, if not identical to the South African putative greening-associated isolate SA07. Two of the nine isolates were eliminated from further consideration by their inability to pass through a 0.45µm millipore filter. Subsequent testing in slot-blot immunoassays with antisera raised to representative greening-associated isolates (Section 3.6) yielded 3 putative ACD-associated isolates serologically related to the greening-associated isolates. These were identified as: AU01, a Gram-variable, small, irregular rod isolated from the midribs of symptomatic leaves collected in Gol Gol (Figure 27a); AU02, a Gram-negative, small, irregular rod isolated from

Nangiloc leaf midribs (Figure 28a) and AU03, a Gram-negative, thin, filamentous rod isolated from Dareton leaf midribs (Figure 29a).

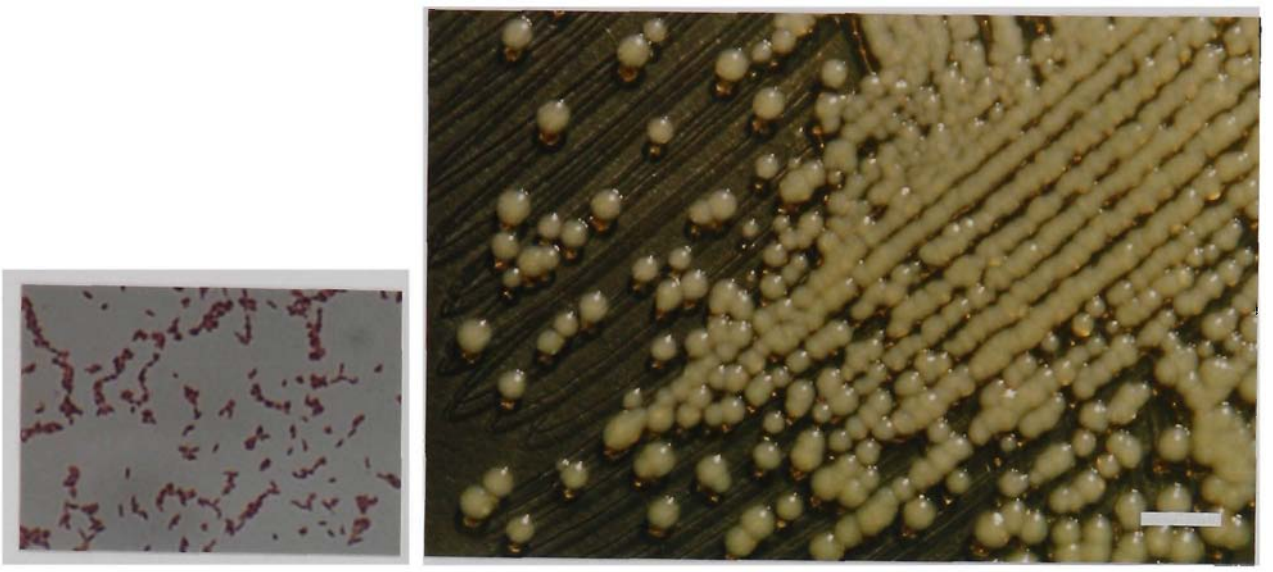
3.4.3 Colony characteristics

Although isolates AU01, AU02 and AU03 were convex, circular and entire morphologically on complete MIG medium at 25°C, the colonies differed considerably in their pigmentation and sizes. AU01 initially grew as a pale yellow colony but subsequently developed a strong yellow pigmentation with subculture (Figure 27b). The AU01 colonies ranged in diameter from 1 - 4mm. AU02, however, grew as small orange/cream pigmented colonies (Figure 28b) approximately 2 - 3mm in diameter. AU03 colonies were initially translucent pin point colonies (Figure 29b) which developed a slight granular whitish appearance with age. Older AU03 colonies measured 1 - 4mm in diameter. The pigmentations of all three colonies became more evident with subculturing.

These putative Australian dieback-associated isolates were further characterized according to their ability to metabolize different carbon sources as described in section 3.3.

3.5 Antisera raised against whole cells

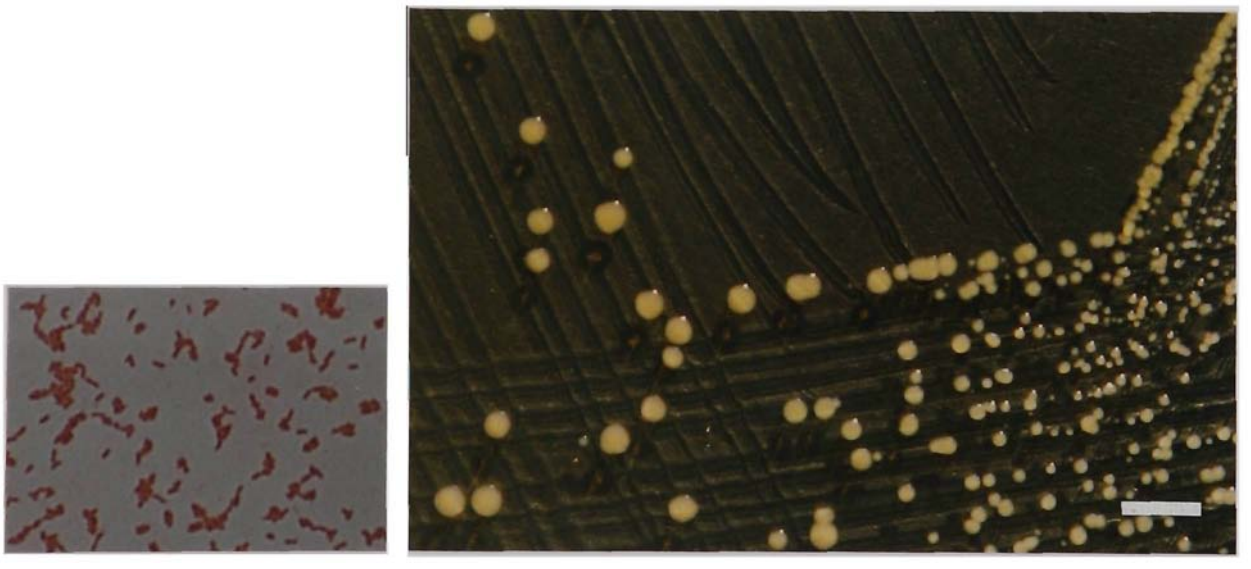
Antisera were raised against the putative greening-associated isolates SA01, SA03, SA07 and RE01 (Section 2.9.1.1). These isolates were chosen as they represent two low temperature isolates from different areas in South Africa (SA01 from Randburg and SA03 from Nelspruit), a high temperature South African isolate (SA07 from Letaba) and a Reunion isolate (RE01) as defined by Mochaba (1988). Furthermore, the serological relationship between the two differently pigmented.



(a) (b)

FIGURE 27 – AU01 colony morphology and Gram stain

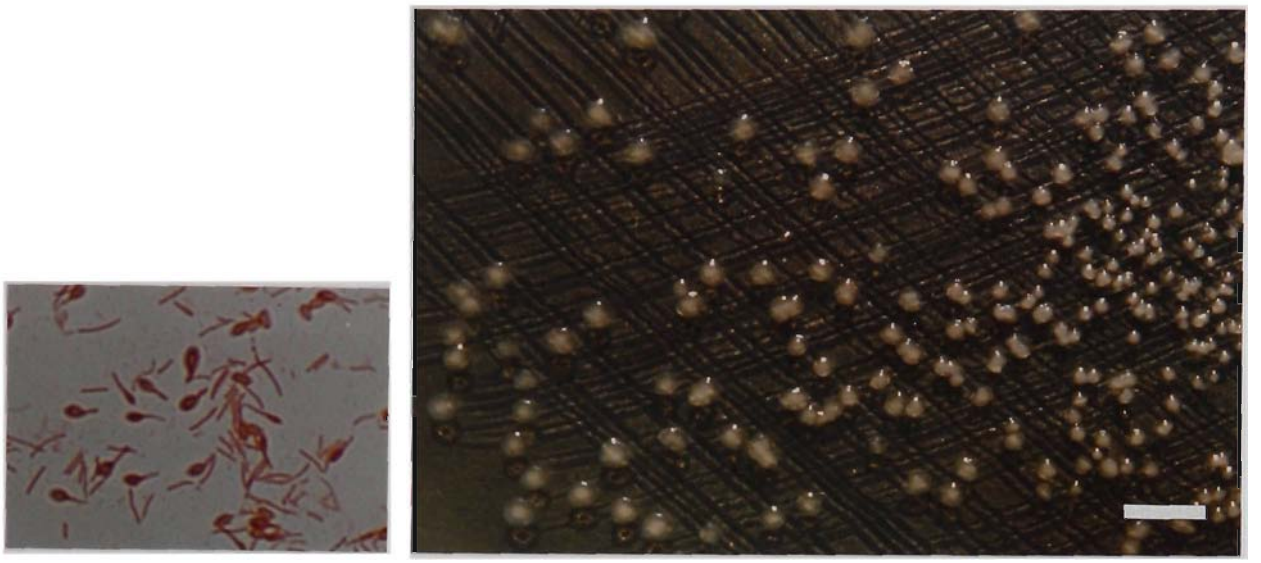
The cells of isolate AU01, staining Gram-negative/positive, were small and irregular (a). (Magnification: 2000x). Colonies (3 days old and grown at 25ºC) appeared translucent and cream with only a hint of yellow (b), developing a stronger yellow pigmentation with growth. (Bar represents 5mm).



(a) (b)

FIGURE 28 – AU02 colony morphology and Gram stain

The cells of isolate AU02 were Gram-negative, irregular, rods (a). (Magnification: 2000x). Colony morphology and pigmentation after 3 days of growth at 25ºC is illustrated in (b). The colonies are a distinct deep yolky/yellow colour. (Bar represents 5mm).



(a) (b)

FIGURE 29 - AU03 colony morphology and Gram stain

The cells of isolate AU03 are Gram-negative, long, thin, rods many of which have subterminal spore-like structures (a). (Magnification: 2000x). Colonies after 5 days of growth at 25°C were small, translucent white and granular (b). Pigmentation may become creamier with age and subculturing. (Bar represents 5mm).

colonies of isolate SA03 could be investigated if an antiserum was available to one of them.

The antibody titre was determined for each by means of a slot-blot immunoassay as described in section 2.9.6.1. Applications, 10µl, of ten-fold dilutions of the antigen at an initial concentration of 1×10^9 cells/ml were reacted in a checker-board arrangement with two-fold dilutions of the corresponding antisera. For all four antisera tested, an antibody dilution of at least 1/2,048 reacted with a 1×10^6 cell/ml concentration of the homologous antigen. The corresponding pre-immune sera at an antibody dilution of 1/512 reacted with the homologous antigens only at a concentration of $1 \times 10^8 - 1 \times 10^9$ cells/ml. No reaction was evident with the pre-immune sera at an antibody dilution of 1/2,048 and antigen concentration of 1×10^6 cells/ml. Although the antibody titres for the four sera tested were similar, a more intense colour development in the slot-blot immunoassay occurred with the anti-RE01 serum and homologous antigen. This antigen and antiserum have consequently been favoured in subsequent serological assays.

The IgG fraction of each serum was purified (Section 2.9.3) and used at a concentration of 10µg/ml in slot-blot immunoassays and at a concentration of 10µg/ml and/or 1µg/ml in western blots.

The above slot-blot immunoassays were repeated using the affinity purified anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG at a 10µg/ml IgG concentration. Each of the IgG's were reacted with 10µl applications of 10-fold dilutions of the homologous antigen at an initial concentration of 1×10^9 cells/ml. Both anti-SA03 IgG and anti-RE01 IgG reacted with 1×10^6 cells/ml (effectively 1×10^4 cells per slot on the nitrocellulose membrane). Anti-SA01 IgG reacted with 1×10^7 cells/ml (effectively 1×10^5 cells/spot) and anti-SA07 IgG, 1×10^5 cells/ml (effectively 1×10^3 cells per spot). The pre-immune IgG of both anti-SA01 and

anti-RE01 did not react with the homologous antigen at a concentration lower than 1 x 10^9 cells/ml. Anti-SA03 pre-immune IgG reacted with the homologous antigen at a concentration of 1 x 10^7 cells/ml. No pre-immune serum was available for the rabbit immunized with SA07.

3.6 Detection of the putative greening/dieback-associated isolates and laboratory cultures with the anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG in slot-blot immunoassays

Having established that the anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG reliably reacted with the corresponding homologous antigen (Section 3.5), other available putative greening/dieback isolates were subsequently screened for reactivity.

Furthermore, the reaction, if any, of several laboratory strains including A. radiobacter (identified as possibly related to the putative greening isolates by API assay by Mochaba,1988), E. coli and B. subtilis (a commonly occurring Gram-negative and Gram-positive bacterium respectively), C. michiganense and C. insidiosum (identified in the Biolog metabolic test in section 3.3 as possibly being related to the putative greening-associated isolates) and P. aeruginosa and P. fluorescens (two members of the RNA group 1 pseudomonads) was investigated.

Positive reactions were scored according to the intensity of the AP/substrate colour reaction on the blots.

The putative greening/dieback-associated bacterial isolates and bacterial cell concentrations were adjusted to 1 x 10^8 cells/ml of which 10µl volumes were spotted onto the nitrocellulose membrane and subsequently reacted with the purified IgG's at 10µg/ml as described in section 2.9.6.1.

Anti-SA01 IgG reacted with SA01, SA03, SA05, RE01, TA01, TA02, AU01, AU02, US04, E. coli and B. subtilis. A strong colour reaction occurred with SA06. The pre-immune IgG reacted with the same intensity as the immune IgG with E. coli and B. subtilis and less strongly than the immune IgG with SA06.

Anti-SA03 IgG reacted strongly with SA01, SA03, SA05, SA06, RE01, TA01, TA02, AU01, AU02 and US04 (Figure 30). A weaker reaction occurred with SA07, SA11, AU03, B. subtilis and E. coli. The strong pre-immune IgG reaction with SA06 and weaker reactions with SA01, SA03, SA05, RE01, TA01, TA02, and E. coli, would suggest the occurrence of possible non specific reactiions.

Anti-RE01 IgG reacted strongly with SA01, SA03, SA05, SA06, RE01, TA01, TA02, US04 and B. subtilis. The pre-immune IgG reaction was very weak with SA01, SA03, SA05, SA06, RE01, TA01, TA02, B. subtilis.

Anti-SA07 IgG reacted strongly with SA07, AU03 and B. subtilis. No pre-immune serum from this rabbit was available.

It is apparent that the anti-SA01, anti-SA03, and anti-RE01 IgG are not only able to detect the homologous antigen, but also cross react with the several putative greening/dieback-associated isolates. One problem that was evident however, was the ability of the pre-immune IgG's to react with some of the greening/dieback-associated isolates and B. subtilis at the same concentrations although the intensity of the reactions were very much lower (Figure 30). The bacterial cell sizes of laboratory cultures such as B. subtilis are not only variable but also larger than the cells of the putative greening/dieback-associated isolates. Consequently, the preparations spotted were standardized according to the absorbance of the suspension rather than the cell concentration. Equal proportions of epitopes are

detected in this way by the IgG's, which reduces not only any non specific binding, but also any cross reactions with the pre-immune IgG.

Bacterial suspensions were adjusted to an absorbance of 0.100 ± 0.002 at 550nm and diluted 1/10 before 10µl volumes were spotted and reacted with anti-SA01, anti-SA03 and anti-RE01 IgG at 10µg/ml. Similar reactions occurred with the three IgG's (Figure 31). Positive reactions occurred with SA01, SA03, SA05, SA06, RE01, TA01, TA02 and US04. Very faint reactions occurred with US07, US08 and C. michiganense. The corresponding pre-immune IgG for anti-SA01 did not react with any of the isolates while only very faint reactions occurred with the corresponding pre-immune IgG's for anti-SA03 and anti-RE01 when reacted with the SA01, SA03, SA05, SA06, TA01, TA02, and US04 isolates.

Anti-SA07 IgG reacted strongly with SA07 and AU03 while weaker reactions occurred with SA09, SA10, US07, US08 and C. michiganense (Figure 32). No corresponding pre-immune observations were available.

The cross reactions observed in the above dot blots between the anti-SA01, anti-SA03, anti-SA07 and anti-RE01 purified IgG's and the putative greening/dieback-associated isolates, suggest that many of the isolates are serologically related. Indeed, two serological groups appear to have been established. One group includes isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02, US04, AU01 and AU02 that react similarly with anti-SA01, anti-SA03 and anti-RE01 IgG. The other group contains the SA07 and AU03 isolates that react with anti-SA07 IgG. The faint reactions of anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG with US07, US08 and C. michiganense may either be attributed to non-specific binding of the antisera or a weaker serological relationship between these organisms and the isolates in the SA01, SA03, SA05, SA06, SA07, RE01, TA01, TA02, AU01, AU02,

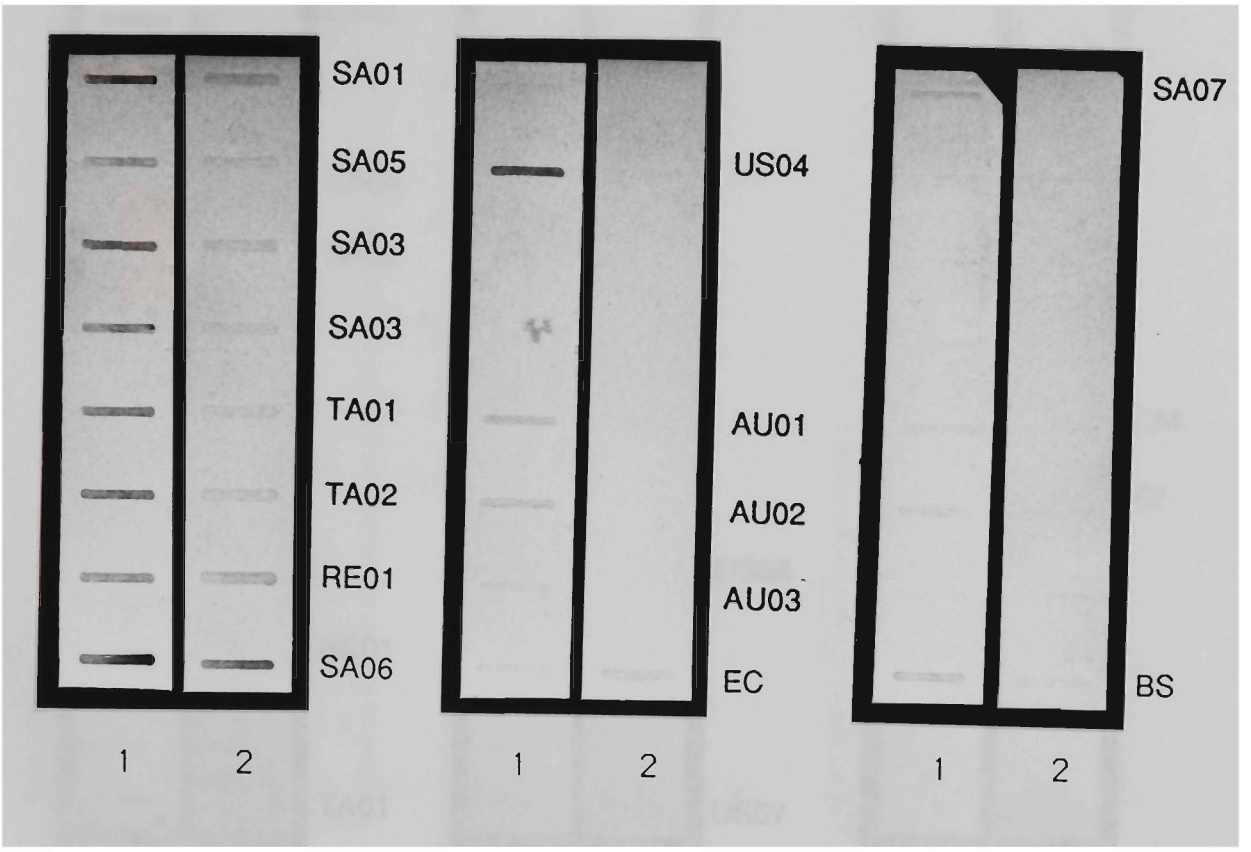


FIGURE 30 - Slot-blot immunoassay for anti-SA03 reacted with the putative greening/dieback-associated isolates and laboratory bacterial strains

Bacterial suspensions, 10µl, adjusted to a concentration of 1 x 10^8 cells/ml were spotted and reacted with both anti-SA03 IgG at 10 µg/ml (Lane 1) and the corresponding pre-immune IgG at the same concentration (Lane 2). (EC) and (BS) represent E. coli and B. subtilis respectively.

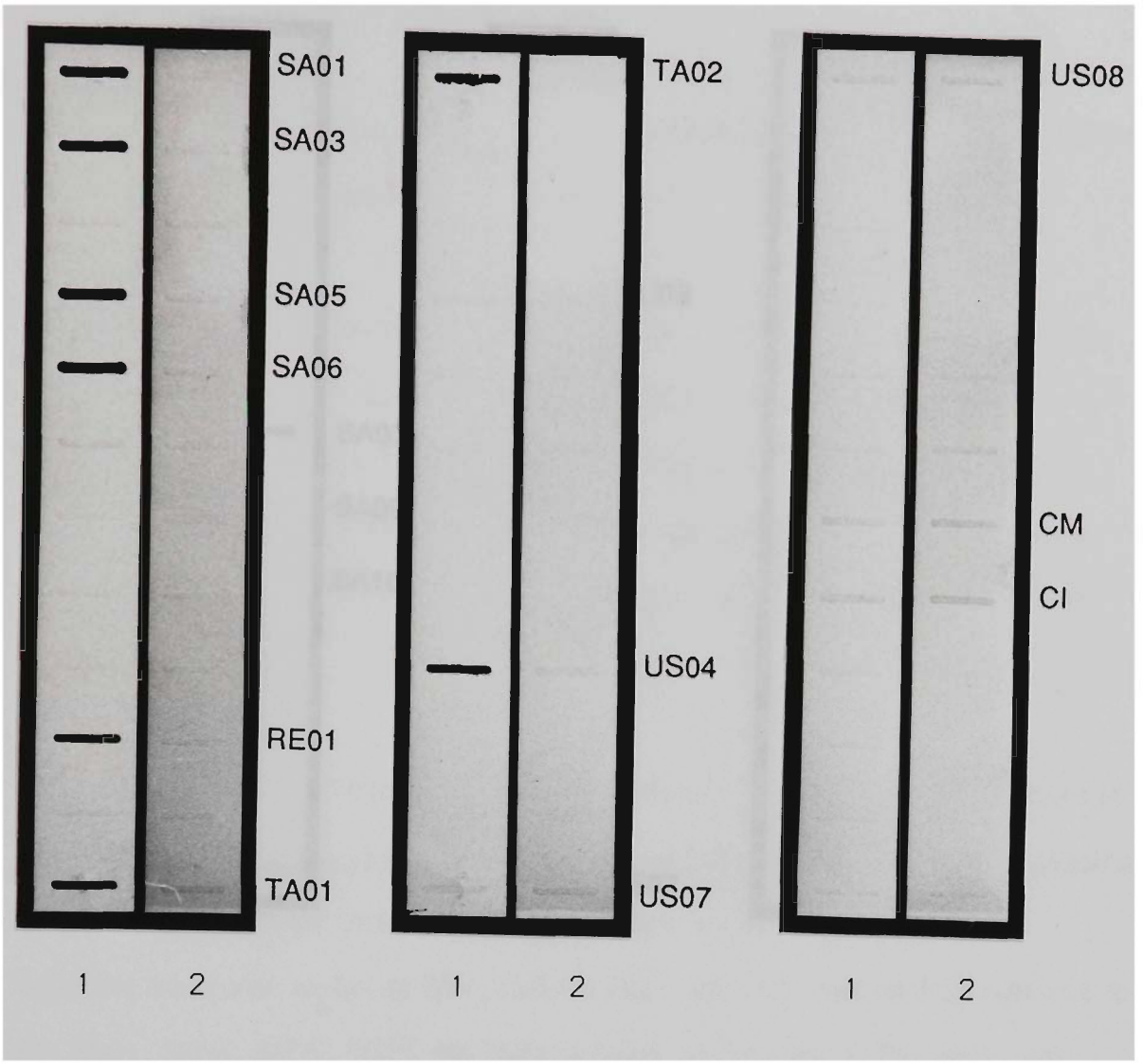


FIGURE 31 - Slot-blot immunoassay for anti-SA03 IgG reacted with the putative greening/dieback-associated isolates and laboratory bacterial strains

Bacterial suspensions, 10µl, adjusted to an absorbance of 0.100 ± 0.002 at 550nm reacted with anti-SA03 IgG at 10µg/ml is illustrated (lane 1). The corresponding pre-immune reactions are illustrated in lane 2. Similar serological reaction patterns were detected with anti-SA01 and anti-RE01 IgG's at the same concentrations. (CM) and (CI) represent Clavibacter michiganense subsp. michiganense and Clavibacter michiganense subsp. insidiosum respectively.

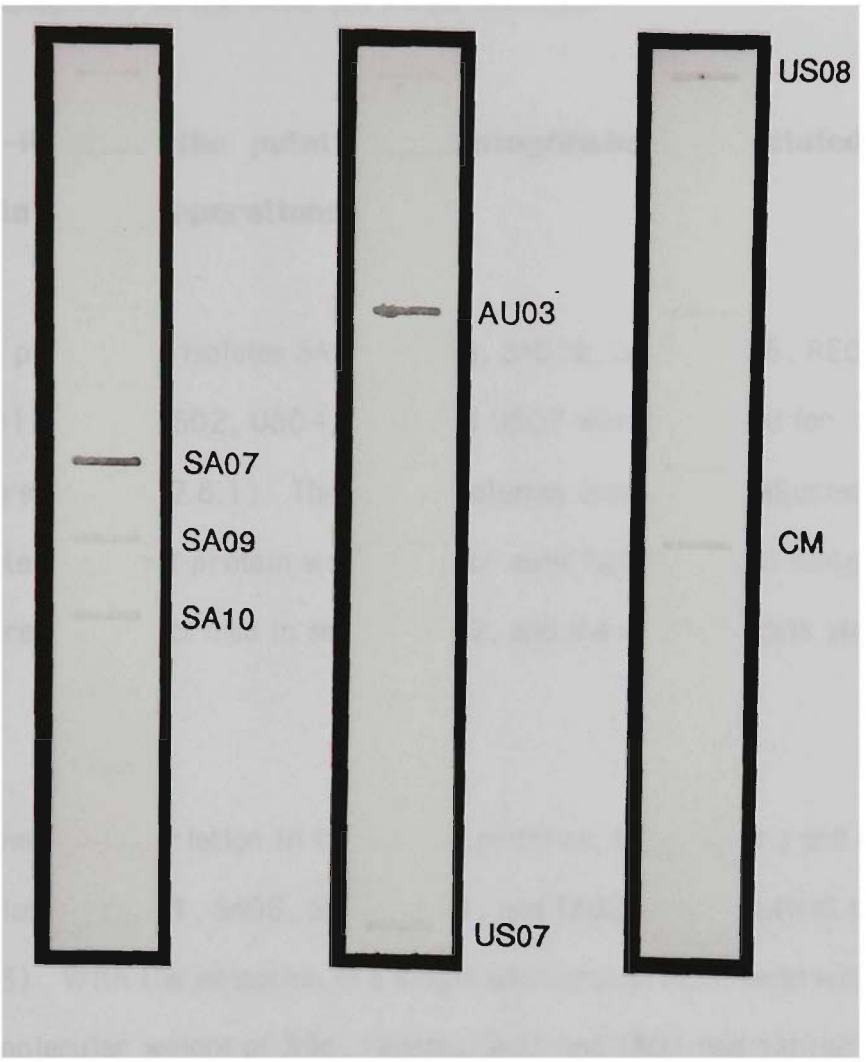


FIGURE 32 - Slot-blot immunoassay for anti-SA07 IgG reacted with the putative greening/dieback-associated isolates and laboratory bacterial strains

Bacterial suspensions, 10µl, adjusted to an absorbance of 0.100 ± 0.002 at 550nm were reacted with 10µg/ml of anti-SA07 IgG. No pre-immune reaction was available. (CM) represents Clavibacter michiganense subsp. michiganense.

AU03 and US04 group. Similarly the SA09 and SA10 isolates may be distantly related serologically to the SA07 and AU03 cultures.

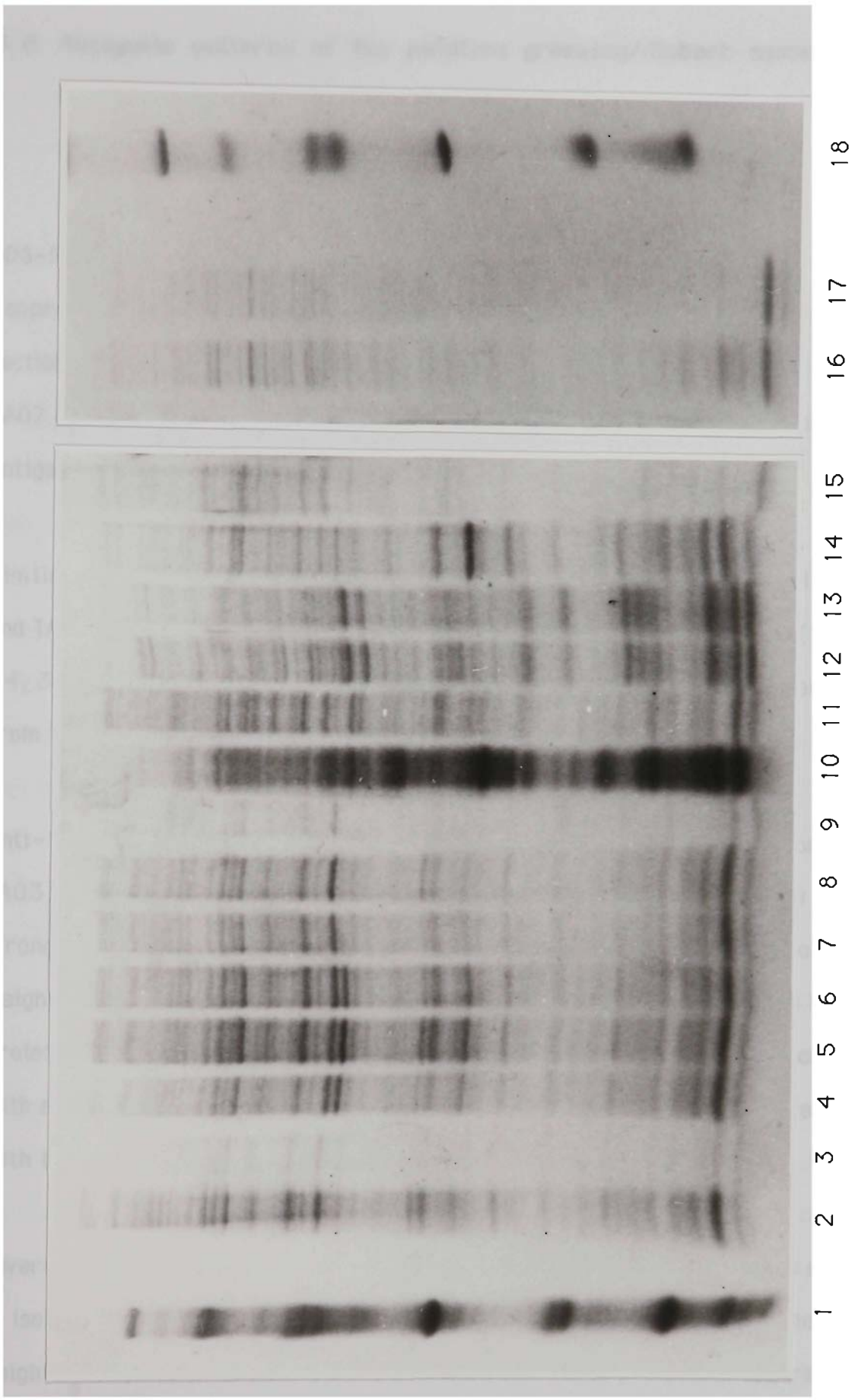
3.7 SDS-PAGE of the putative greening/dieback-associated bacterial whole cell preparations

Whole cell proteins of isolates SA01, SA03a, SA03b, SA05, SA06, RE01, TA01, TA02, AU01, AU02, US02, US04, US05 and US07 were prepared for electrophoresis (Ref. 2.8.1). The sample volumes loaded were adjusted so that approximately 25µg of protein was loaded for each isolate. These samples were electrophoresed as described in section 2.8.2, and the resulting gels stained and analysed.

Although there was variation in the protein patterns, the numbers and positions of bands for isolates SA01, SA05, SA06, RE01, and TA02 were identical to one another (Figure 33). With the exception of a single additional protein band with an apparent molecular weight of 39K, isolates SA03 and TA01 had similar patterns to the SA01, SA05, SA06, RE01 and TA02 isolates. Furthermore, the whole cell protein patterns for the SA03 white colony and yellow colony variants were exactly the same (Figure 33). Protein patterns of isolates AU01 and US04 were similar to those of isolates SA01, SA05, SA06, RE01 and TA02. The pattern observed for AU02 closely resembled the patterns for C. michiganense and C. insidiosum. As the protein patterns resulting from 25µg of protein of these latter organisms were very faint, when stained with Coomassie, the gels were repeated with 50µg to allow adequate comparison. None of the patterns of the greening/dieback-associated bacterial isolates were the same or similar to those (resulting from 25µg protein loaded) of A. radiobacter, B. subtilis, E. coli, P. aeruginosa and S. typhimurium.

FIGURE 33 - Whole cell protein patterns

Total cell protein samples, 25µg, for several isolates were loaded, electrophoresed on a 10% - 17.5% gradient gel and subsequently stained. The patterns of preparations from isolates SA01, SA05, TA02, RE01 and SA06 are identical (Lanes 2, 3, 7, 8, and 9 respectively). Also, the patterns of similar preparations from isolates TA01 (Lane 6) and the white and yellow SA03 isolates - SA03a (Lane 5) and SA03b (Lane 4) respectively, are identical. With the exception of a single 38K - 39K protein, these are similar to the SA01, SA05, TA02, RE01 and SA06 patterns. The protein patterns of isolates US04 and AU01 (Lanes 11 and 14 respectively) are also similar to isolates SA01, SA05, TA02, RE01, SA06, US04. The protein pattern of isolate AU02, although different from the isolates above, is comparable to the patterns obtained for both C. michiganense (Lane 16) and C. insidiosum (Lane 17) for which 50µg of protein was loaded/well. Other isolates include: US02 (Lane 10), US05 (Lane 12) and US07 (Lane 13). Lanes 1 and 18 contain the low molecular weight markers.


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
94.0K
67.0K
43.0K
30.0K
20.1K
14.4K

3.8 Antigenic patterns of the putative greening/dieback-associated bacterial isolates reacted with anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG

SDS-PAGE gels resulting from electrophoresis of whole cell preparations (approximately 50µg of protein was loaded per well) were blotted as described in section 2.9.7. The blots were reacted with purified anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG at a concentration of 10µg/ml. The resulting antibody-antigen reactions visualized as described in 2.9.6.1.

Similar patterns were obtained for isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 when reacted with the anti-SA01, anti-SA03 and anti-RE01 IgG (Figures 34, 35 and 36 respectively). Protein bands were also detected in the preparations from isolates AU01 and US04.

Anti-SA01 IgG reacted with 5 cell proteins in the whole cell preparations for SA01, SA03, SA05, SA06, RE01, TA01 and TA02 (Figure 34). The anti-SA01 IgG reacted strongly with a 38K - 40K protein and weakly with proteins of apparent molecular weights of 79K, 75K, 36K and 23K. Weak reactions also occurred with two US04 proteins of apparent molecular weights of 87K and 35K. Strong reactions occurred with a 37K - 39K protein in both US04 and AU01. None of these proteins reacted with the corresponding pre-immune IgG in western blots.

Several proteins reacted with the anti-SA03 IgG (Figure 35). The proteins detected in isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 had apparent molecular weights: 78K, 75K, 72K, 59K, 51K, 39K and 35K. Three proteins of apparent molecular weights of 80K, 37K and 35K from isolate US04 and only a single 37K - 38K protein from isolate AU01 reacted with the antiserum. From the band widths, the 51K, 39K, and 35K bands associated with isolates SA01, SA03, SA05, SA06,

RE01, TA01 and TA02 and the 37K band associated with isolate US04 illustrated in figure 35 could represent two closely positioned proteins. None of these proteins were detected in western blots reacted with the corresponding pre-immune IgG.

Preparations of isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 (Figure 36) contained proteins of apparent molecular weights: 116K, 104K, 90K, 73K, 52K, 39K and 34K when reated with the anti-RE01 IgG. The 116K and 104K proteins also occur in isolate US04. Furthermore, a 37K - 38K protein was detected in both US04 and AU01. Again, there was no reaction with pre-immune IgG.

Comparing the western blots to one another, a single protein band of molecular weight 38K - 39K (Figures 34, 35 and 36) occurs in isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 that reacts with the anti-SA01, anti-SA03 and anti-RE01 IgG's. Similarly, the 37K - 38K protein of isolate US04 (Figures 34, 35 and 36) and the 37K - 39K protein of isolate AU01 (Figures 34, 35 and 36) react with these three IgG's.

The anti-SA07 IgG reacted with several proteins in the SA07 and AU03 whole cell preparations resulting in identical serological reactions for these isolates (Figure 37) distinct from those obtained with the other IgG/isolate reactions. None of the other isolates tested contained proteins that reacted with anti-SA07 IgG except isolate US02 which yielded a single 37K - 38K protein (Figure 38). No reaction with pre-immune IgG was available.

From the above observations it appears that there are two serologically distinct groups. Most of the isolates could be grouped together and include isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02. Isolates AU01 and US04 appear to be serologically related but not serologically identical to this group. A second group

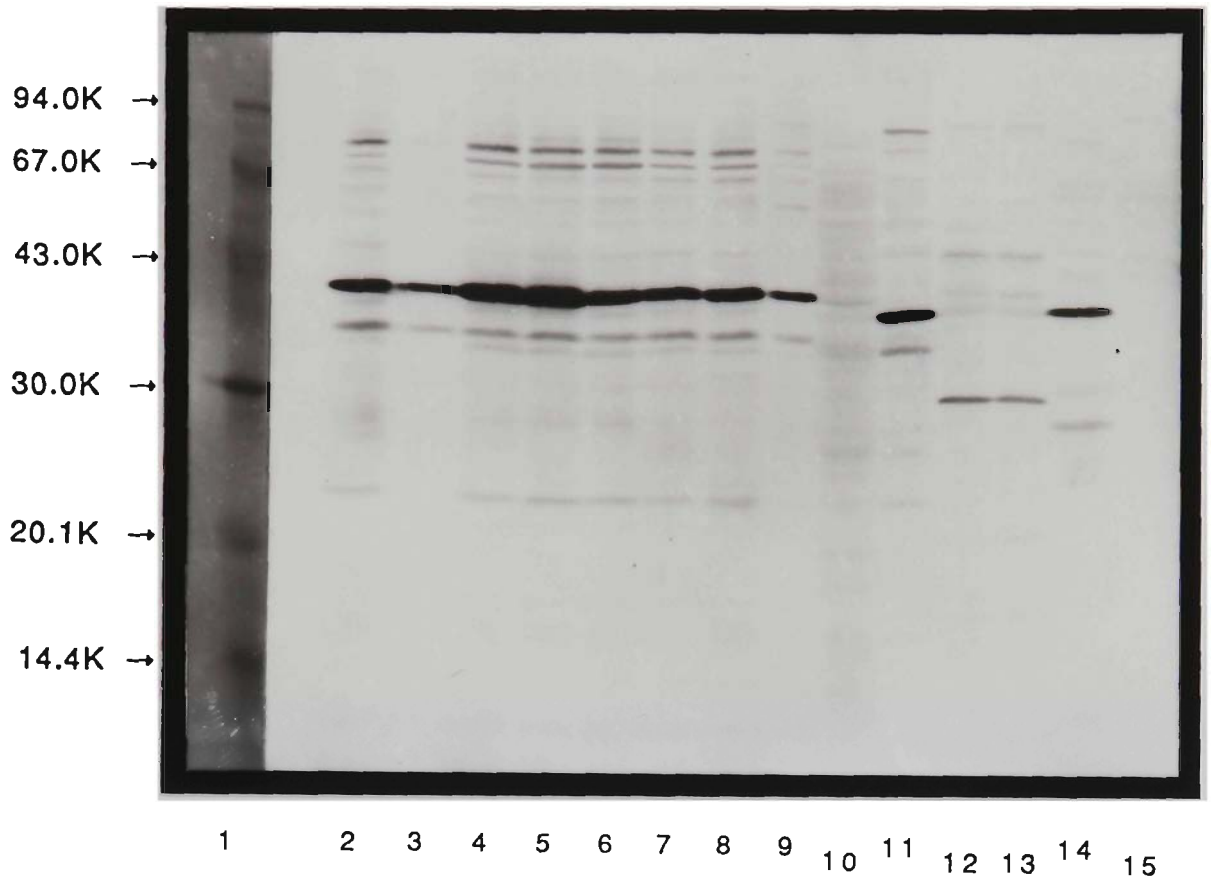


FIGURE 34 - Serological reaction patterns of whole cell protein preparations from putative greening/dieback-associated bacterial isolates with anti-SA01 IgG

The protein bands detected in a western blot resulting from the electrophoretic separation of whole cell samples containing 50µg of protein and reacted with anti-SA01 IgG at 10µg/ml are illustrated. Reactions of preparations from SA01, SA05, SA03b, SA03a, TA01, TA02, RE01, SA06, US04 and AU01 isolates are represented (Lanes 2, 3, 4, 5, 6, 7, 8, 9, 11 and 14, respectively). Other lanes are: US02 (Lane 10), US05 (Lane 12), US07 (Lane 13) and AU02 (lane 15). Lane 1 contains the low molecular weight markers.

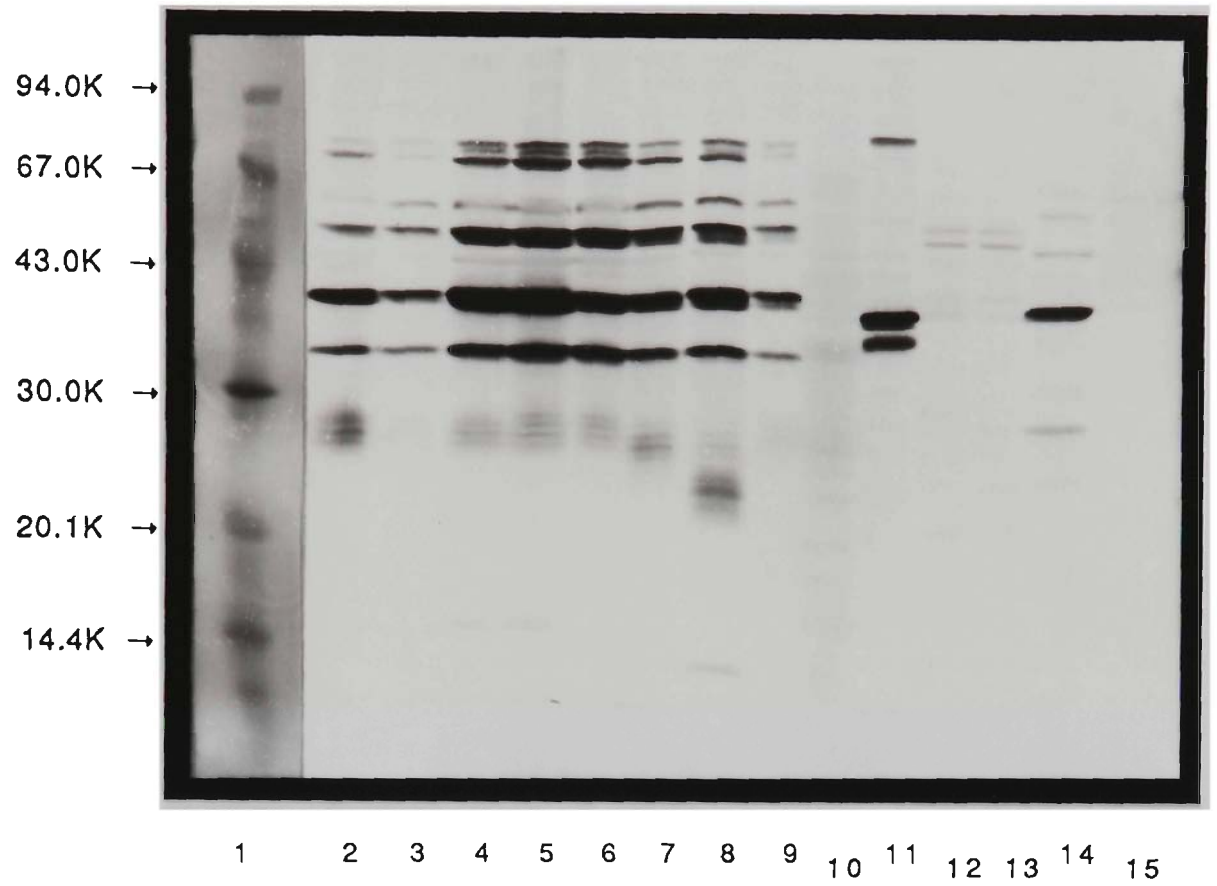


FIGURE 35 - Serological reaction patterns of whole cell protein preparations from putative greening/dieback-associated bacterial isolates with anti-SA03 IgG

The protein bands detected in a western blot resulting from the electrophoretic separation of whole cell samples containing 50µg of protein and reacted with anti-SA03 IgG at 10µg/ml are illustrated. Reactions of preparations from SA01, SA05, SA03b, SA03a, TA01, TA02, RE01, SA06, US04 and AU01 isolates are represented (Lanes 2, 3, 4, 5, 6, 7, 8, 9, 11 and 14, respectively). Other lanes are: US02 (Lane 10), US05 (Lane 12), US07 (Lane 13) and AU02 (lane 15). Lane 1 contains the low molecular weight markers.

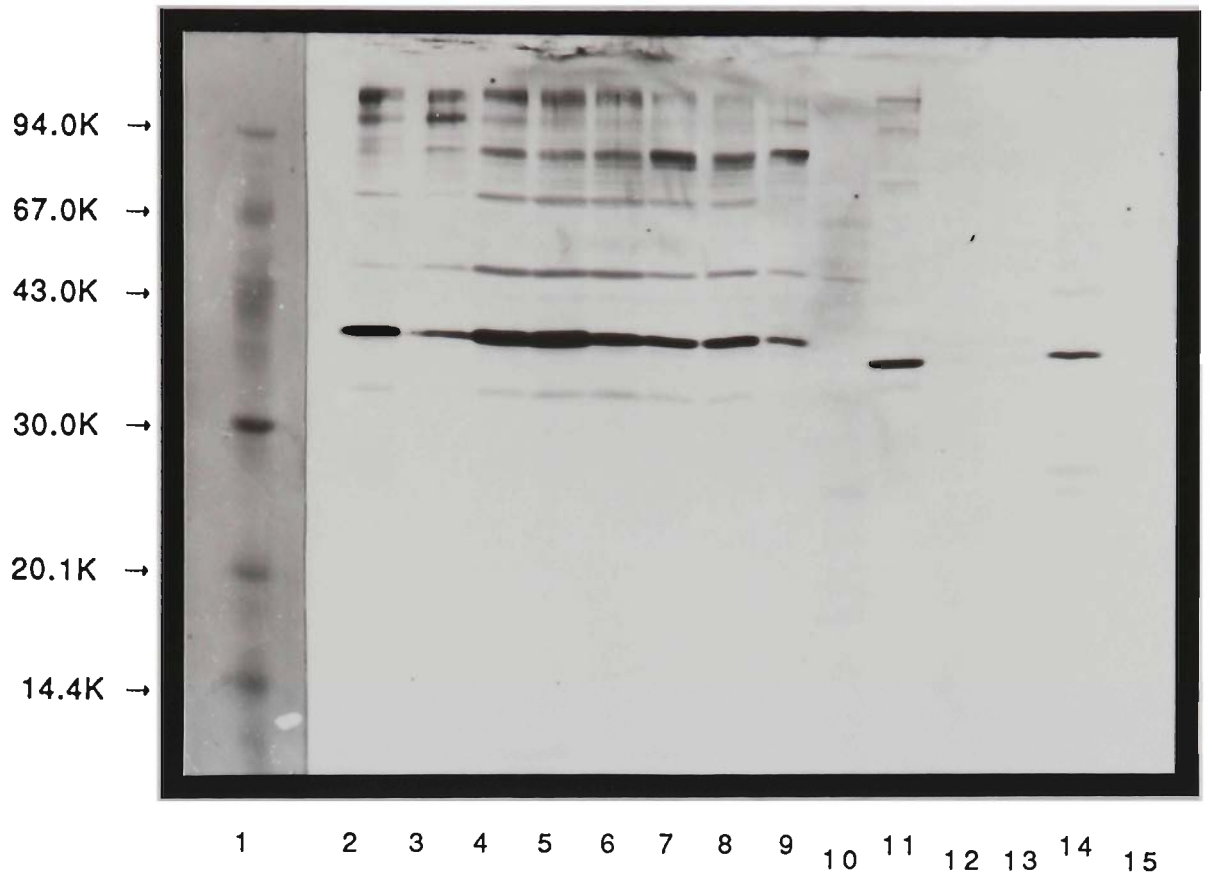


FIGURE 36 - Serological reaction patterns of whole cell protein preparations from putative 'greening/dieback-associated bacterial isolates with anti-RE01 IgG

The protein bands detected in a western blot resulting from the electrophoretic separation of whole cell samples containing 50µg of protein and reacted with anti-RE01 IgG at 10µg/ml are illustrated. Reactions of preparations from SA01, SA05, SA03b, SA03a, TA01, TA02, RE01, SA06, US04 and AU01 isolates are represented (Lanes 2, 3, 4, 5, 6, 7, 8, 9, 11 and 14, respectively). Other lanes are: US02 (Lane 10), US05 (Lane 12), US07 (Lane 13) and AU02 (lane 15). Lane 1 contains the low molecular weight markers.

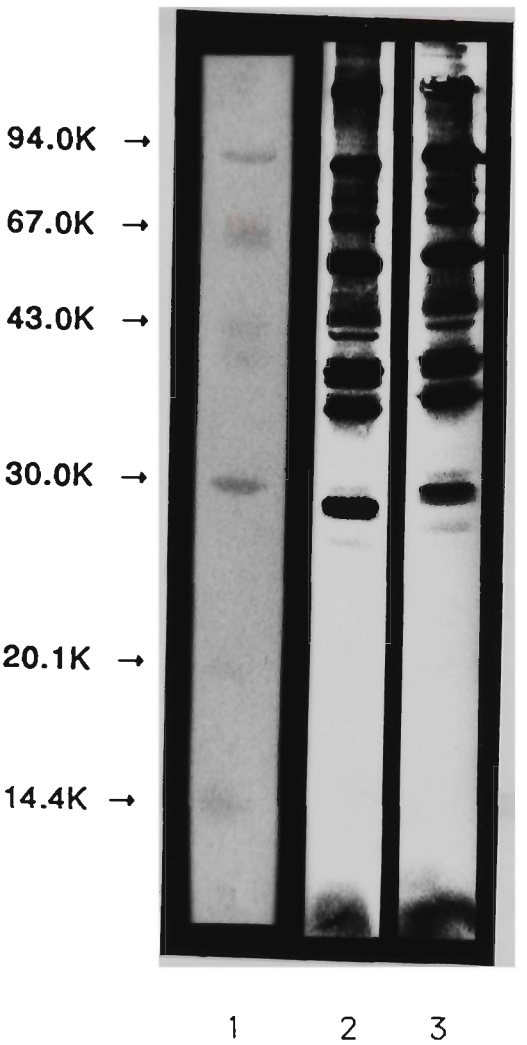


FIGURE 37 - Serological reaction patterns of whole cell protein preparations from isolates SA07 and AU03 with anti-SA07 IgG

The protein bands detected in a western blot following electrophoretic separation of SA07 (Lane 2) and AU03 (Lane 3) whole cell samples containing 25µg of protein and reacted with anti-SA07 IgG at 10 µg/ml are illustrated. Lane 1 contains the low molecular weight markers.

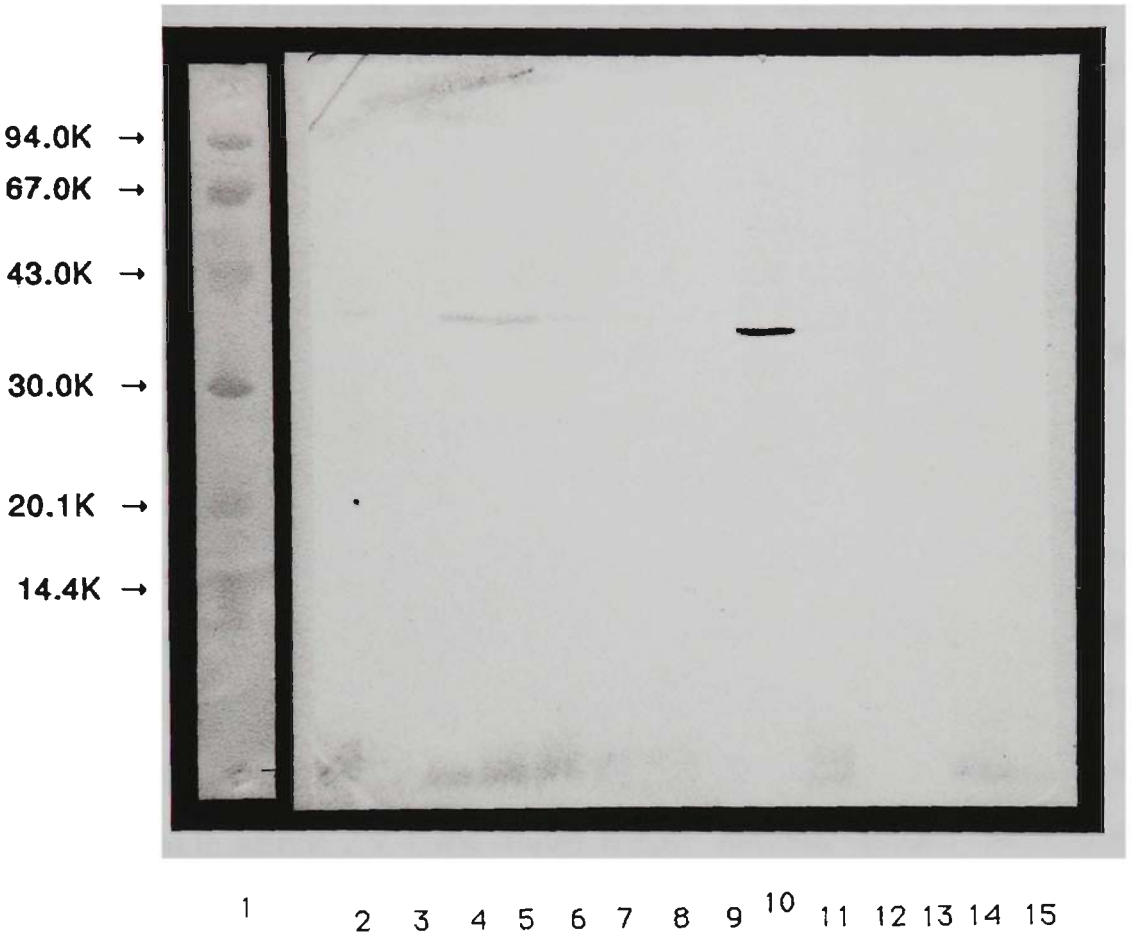


FIGURE 38 - Serological reaction patterns of whole cell protein preparations from putative greening/dieback-associated bacterial isolates with anti-SA07 IgG

The protein bands detected in a western blot folowing electrophoretic separation of whole cell protein preparations containing 50µg of protein and reacted with anti-SA07 IgG at 10µg/ml are illustrated. Only a single protein band was detected in isolate US02 (Lane 10). Other lanes are: SA01 (Lane 2), SA05 (Lane 3), SA03b (Lane 4), SA03a (lane 5), TA01(Lane 6), TA02 (Lane 7), RE01 (Lane 8), SA06 (lane 9), US04 (Lane 11), US05 (Lane 12), US07 (Lane 13), AU01 (Lane 14) and AU02 (lane 15). Lane 1 contains the low molecular weight markers.

contains isolates SA07 and AU03, and US02 may be related. These observations are complemented by the protein pattern observations. In addition, the serological similarities and differences of the isolates are comparable to the similarities and differences among the isolates with respect to the metabolic, growth curve, colony morphology, light microscope and slot-blot results.

3.9 Serological reaction patterns of SA03 and RE01 with antisera 228/12, 228/13, 228/14, 228/15, 228/16 and 228/17

SA03 and RE01 whole cell protein preparations were prepared for electrophoresis as described in 2.8.1. Approximately 50µg of protein was loaded for each isolate and subjected to SDS-PAGE (2.8.2). The resulting gel was blotted (2.9.7) and the blot reacted with the 228/12 - 228/17 antisera obtained from another laboratory as described in section 2.9.4. and diluted 1/250. Antibody-antigen reactions were visualized using labelled goat anti-rabbit IgG as described in 2.9.6.1. The reactions of the preparations from isolates SA03 and RE01 reacted with the antisera were identical (Figure 39).

Antiserum 228/12 reacted with three proteins with apparent molecular weights of 88K, 52K and 43K. The 88K protein also reacted with the 228/13, 228/14 and 228/15 antisera. All six antisera reacted with a 42K - 43K protein. Antiserum 228/13, reacted with an additional four proteins of apparent molecular weights: 67K, 63K, 21K and 20K. With the exception of the 63K protein, and the two low molecular weight proteins, the antigens that reacted with 228/15 were similar to those detected by 228/13. Antiserum 228/14 reacted with four proteins of apparent molecular weights: 88K, 83K, 42K and 37K. Four proteins of apparent molecular weights: 62K, 49K, 42K and 37K reacted with 228/17. Although a high degree of background is evident in the 228/16 blot, a 51K - 52K and 43K - 44K protein band could still be resolved.

It is interesting that the antigenic bands that reacted with the 228/12 - 228/17 antisera are comparable to the antigenic bands detected in the in vitro whole cell preparations reacted with anti-SA01, anti-SA03 and anti-RE01 IgG (Figures 34, 35 and 36); particularly because the 228/12 - 228/17 antisera were raised to proteins in affected plant material that react with UF6 antiserum.

3.10 Antisera against bacterial cell proteins

As several protein bands in isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 reacted with anti-SA01, anti-SA03 and anti-RE01 IgG in western blots, these proteins were used to raise more specific antisera.

Serological patterns for RE01 were compared when western blots were reacted with anti-SA01, anti-SA03 and anti-RE01 IgG at 10 µg/ml. The resulting patterns were different for each IgG preparation although several protein bands reacted commonly (Figure 40). As 90µg of protein was loaded, the patterns for isolate RE01 contained more bands than previously observed (Section 3.8). Furthermore the molecular weights estimated as described in Appendix IX differed slightly due to the variations that occur when gradient gels are prepared. Proteins with apparent molecular weights 81K and 76K, 56K, 42K, 36K and 23K were prepared for immunization as described (Section 2.9.1.2). The 81K protein corresponds to the 79K band that reacted with anti-SA01 IgG (Figure 34) and the 78K band that reacted with anti-SA03 IgG (Figure 35) and not previously observed with anti-RE01 IgG (Figure 36); the 76K, 42K and 36K protein bands correspond to the protein bands that previously reacted with anti-SA01 (Figure 34), anti-SA03 (Figure 35) and anti-RE01 IgG (Figure 36) in the apparent molecular weight ranges of 73K - 75K, 38K - 40K and 34K - 36K respectively; the 56K protein corresponds to the 51K and 52K protein bands that previously reacted with anti-SA03 (Figure 35) and anti-RE01 IgG (Figure 36) respectively; and the 23K

FIGURE 39 - Serological reaction patterns for isolates SA03 and RE01 with antisera raised against proteins that reacted in western blots of greening affected tissue

The reaction of antisera raised against proteins which reacted in greening affected tissue by UF6 antiserum in western blots, to SA03 and RE01 whole cell protein profiles (Lanes 2 and 3 respectively) is illustrated. Antisera 228/12, 228/13, 228/14, 228/15, 228/16 and 228/17 reactions are represented by blots (a), (b), (c), (d), (e) and (f), respectively. The sera were diluted 1/250. Samples containing 50µg of whole cell protein were loaded for both SA03 and RE01. Lane 1 contains the low molecular weight markers.


94.0K
67.0K
43.0K
30.0K
20.1K
14.4K
1 2 3 2 3 2 3 2 3 2 3 2 3
(a) (b) (c) (d) (e) (f)

protein corresponds to the 23K protein band previously reacted with anti-SA01 IgG (Figure 34). For immunization the 81K and 76K proteins were pooled and coded as (A), the 56K protein was coded as (B), the 42K as (C), the 36K as (D) and the 23K as (E).

After the immunization protocol was completed test bleeds were performed and the sera tested in western blots for the reactivity with the protein bands used to immunize the rabbits.

Of the five sera raised, only the anti-C serum raised was specific to, and reacted strongly with, a 38K - 40K protein (Figure 41) when a RE01 whole cell protein profile was reacted in a western blot with the antiserum diluted 1/500. This protein band corresponded to the 42K protein band originally immunized. No serological reactivity of the 38K - 40K protein occurred with the pre-immune serum, used at the same dilution, from the animal in which the anti-C serum was raised. The pre-immune sera from animals injected with A, B, D and E protein bands, reacted with protein bands that reacted with the corresponding specific antisera. Anti-C serum was subsequently fractionated according to the procedure described in section 2.9.3 and the purified IgG further used at a concentration of either 10µg/ml or 1µg/ml. The anti-C IgG was highly reactive as the 39K - 40K protein could still be detected at an IgG concentration of 0.01 µg/ml in western blots.

3.10.1 Serological reaction patterns of the putative greening/ dieback-associated bacterial isolates reacted with purified anti-C IgG in western blots

Western blots resulting from gels loaded with 35µg of protein/well of SA01, SA03, SA05, SA06, RE01, TA01, TA02, US04 and AU01 whole cell protein preparations,

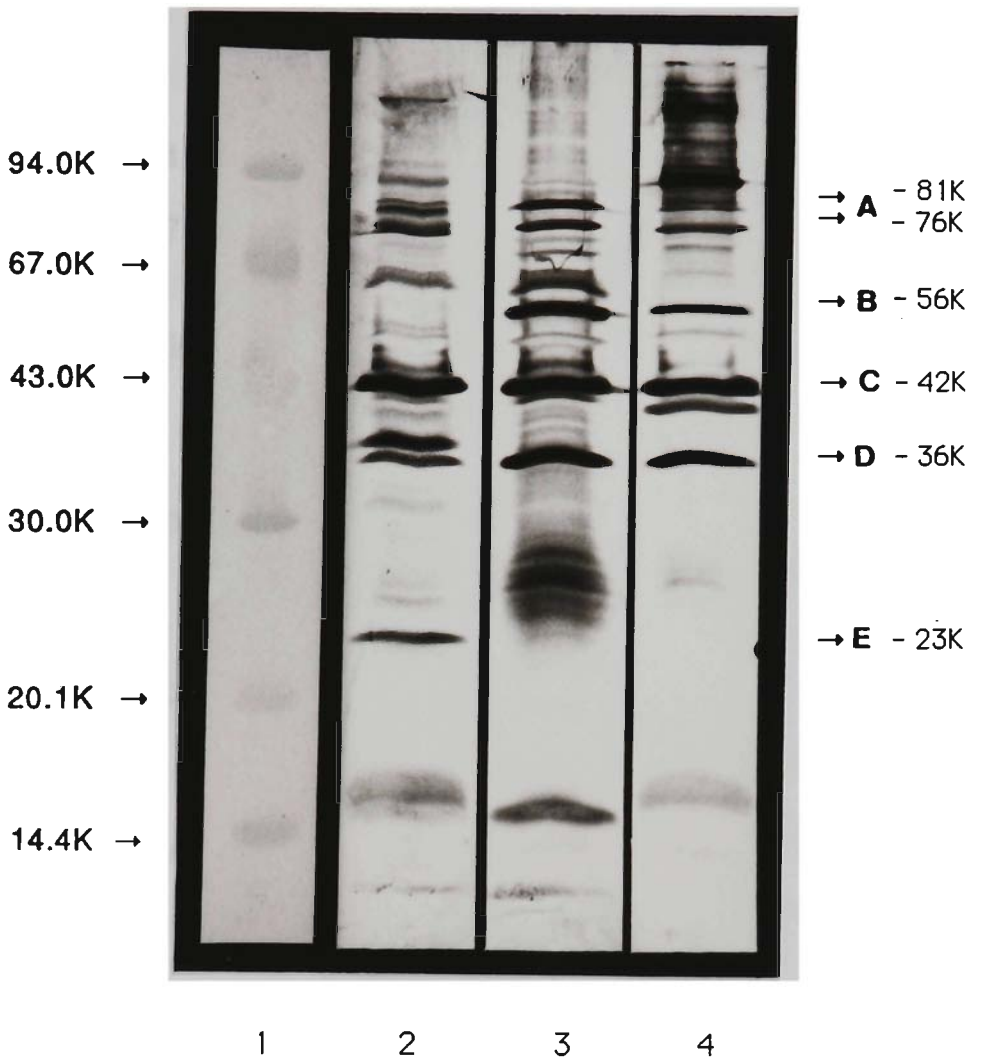


FIGURE 40 - Serological patterns of whole cell protein preparations of isolate RE01 reacted with anti-SA01, anti-SA03 and anti-RE01 IgG

Whole cell, western blot, serological reactions of isolate RE01 reacted with anti-SA01 (Lane 2), anti-SA03 (Lane 3) and anti-RE01 (Lane 4) IgG are compared. The 81K and 76K proteins were pooled and coded as (A), the 56K protein was coded as (B), the 42K as (C), the 36K as (D) and the 23K as (E). Approximately 90µg of RE01 whole cell protein was loaded in each well. The IgG concentration was 10µg/ml. Lane 1 contains the low molecular weight markers.

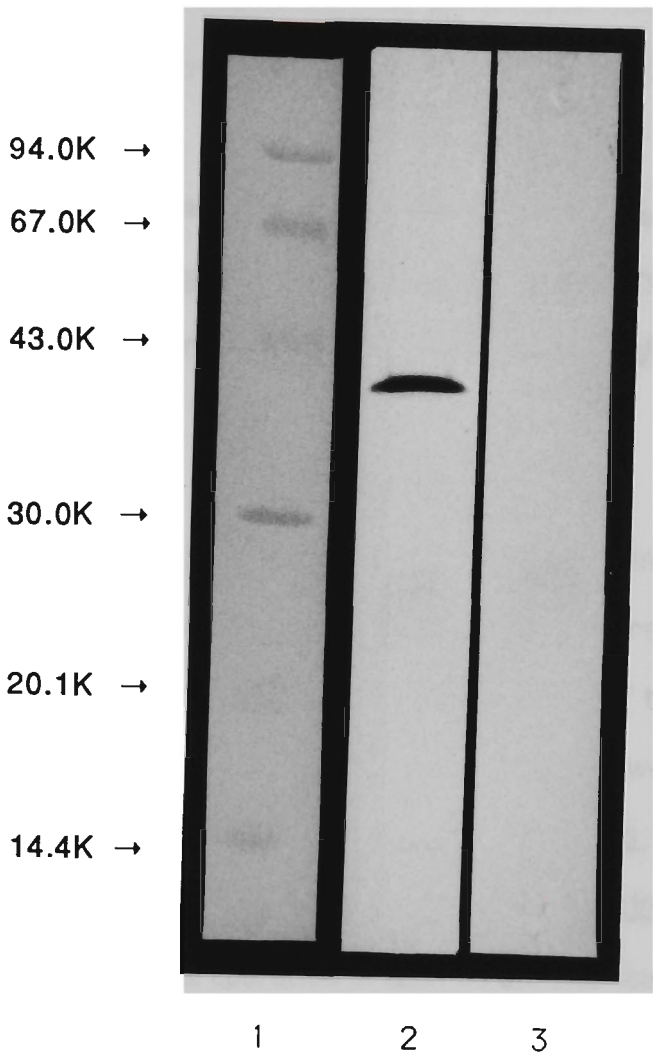


FIGURE 41 - Serological pattern of a whole cell protein preparation of isolate RE01 reacted with anti-C serum

A western blot of isolate RE01 whole cell proteins showing the specificity of the anti-C serum (diluted 1/500) for the 39K protein to which it was raised (Lane 2). No pre-immune reaction is observed (Lane 3) at the same dilution. 67.5µg of isolate RE01 whole cell protein was loaded in each well. Lane 1 contains the low molecular weight markers.

were reacted with purified anti-C IgG at 0.1µg/ml. A single 38K - 39K protein was specifically recognized in preparations from all of these isolates (Figure 42). No reaction was evident for the corresponding pre-immune IgG at 0.1µg/ml.

3.10.2 Serological reaction patterns of B. subtilis, C. insidiosum, C. michiganense, E. coli, P. aeruginosa, P. fluorescens, and S. typhimurium, reacted with purified anti-C IgG in western blots

The anti-C specificity for a single protein band was determined in section 3.10 and the subsequent reaction of this protein in the several putative greening/dieback-associated isolates recorded in section 3.10.1. The specificity of the anti-C IgG for the putative greening/dieback-associated isolates and its use in western blots was investigated by reacting whole cell protein profiles of B. subtilis, C. insidiosum, C. michiganense, E. coli, P. aeruginosa, P. fluorescens, and S. typhimurium. Approximately 25µg of protein preparation of each strain was loaded, electrophoresed and blotted as described in sections 2.8.2 and 2.9.7, respectively. Protein bands reacted (Section 2.9.6.1) with anti-C IgG and corresponding pre-immune IgG, both at a concentration of 1.0µg/ml.

The serological reaction patterns for these strains reacted with anti-C IgG were identical to the corresponding pre-immune pattern (Figure 43). Protein bands were not detected for C. insidiosum and C. michiganense with either the anti-C or pre-immune IgG; only a single protein was detected in B. subtilis; and similar patterns were observed for E. coli, P. aeruginosa, P. fluorescens, and S. typhimurium with both the anti-C or pre-immune IgG. Of special interest were two protein bands of apparent molecular weights: 40K and 38K, detected in the P. fluorescens pattern as these were in the same range as the 38K protein detected by anti-C IgG in the putative greening/dieback-associated bacterial isolate protein

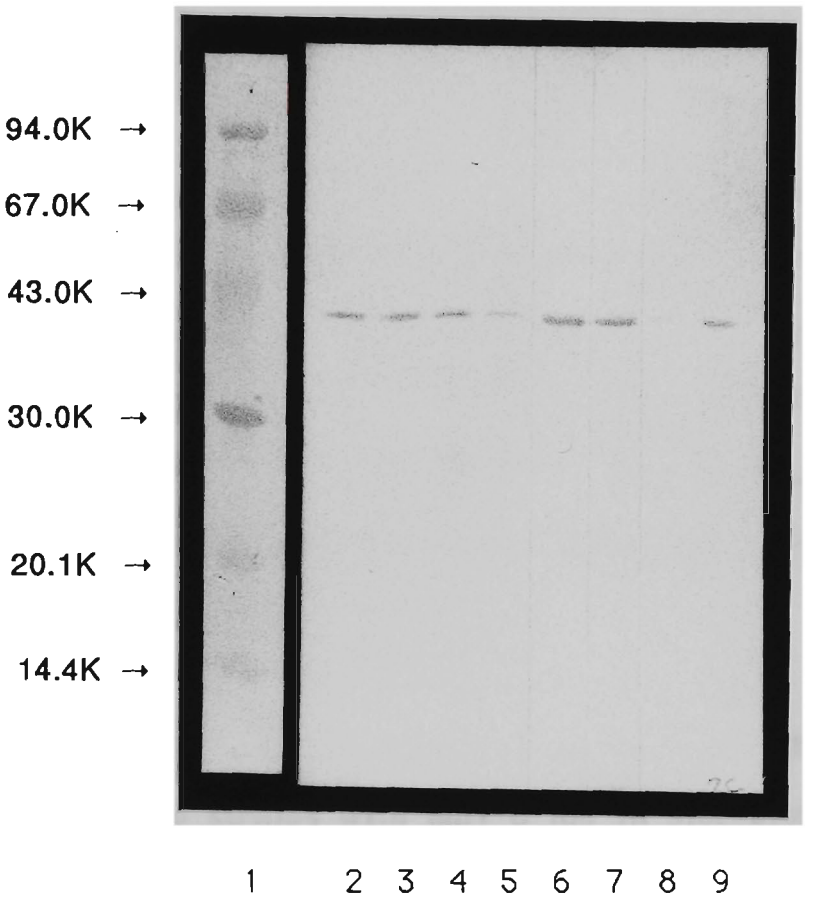


FIGURE 42 - Serological reaction patterns of whole cell protein preparations of putative greening/dieback-associated bacterial isolates reacted with anti-C IgG

The protein band serological reaction patterns observed with the anti-C IgG in western blot reactions with isolates TA01, TA02, SA01, SA05, SA03b, SA03a, AU01 and US04 are indicated (Lanes 2, 3, 4, 5, 6, 7, 8 and 9, respectively). Only a single protein with an apparent molecular weight of 38K - 39K reacted with anti-C IgG at a concentration of 0.1µg/ml. No reaction was evident with the corresponding pre-immune IgG at the same concentration. Approximately 35µg whole cell protein was loaded per well. Lane 1 contains the low molecular weight markers.

patterns (Section 3.9.1). Both the 40K and 38K bands, however, also reacted with the purified pre-immune IgG which did not react with the 38K - 39K protein band in the putative greening/dieback-associated bacterial isolate protein patterns.

Cross absorbing both the anti-C IgG and the corresponding pre-immune IgG with whole P. fluorescens cells (Section. 2.9.5.1) had no effect on the reaction of the 38K - 39K protein band in the putative greening/dieback-associated bacteria patterns with the anti-C IgG while the reaction of the P. fluorescens 38K protein was eliminated (Figure 44). The reaction of the 40K protein in the P. fluorescens samples was not affected by cross absorbing the anti-C IgG or corresponding pre-immune IgG with whole P. fluorescens cells.

3.10.3 Reaction of the putative greening/dieback-associated isolates and laboratory strains with anti-C IgG in slot-blot immunoassays

Slot-blot immunoassays using the more specific anti-C IgG did not differentiate between bacterial isolates. Contrary to the protein specific reaction observed for the putative greening/dieback-associated bacterial isolates reacted with anti-C IgG in western blots (Section 3.10.1), both the anti-C IgG and corresponding pre-immune IgG were found to react equally in slot-blot immunoassays of whole cell suspensions.

This problem was first observed in a slot-blot immunoassay in which the anti-C IgG at 10µg/ml reacted strongly with isolates SA01, SA05, TA01, TA02, AU01 and C. michiganense bacterial cell suspensions, 10µl applications, at a concentration of 1 x 10^8 cells/ml. Slightly weaker reactions occurred with RE01, AU02, AU03, C. insidiosum, P. fluorescens and S. typhimurium. A faint reaction was evident for P.

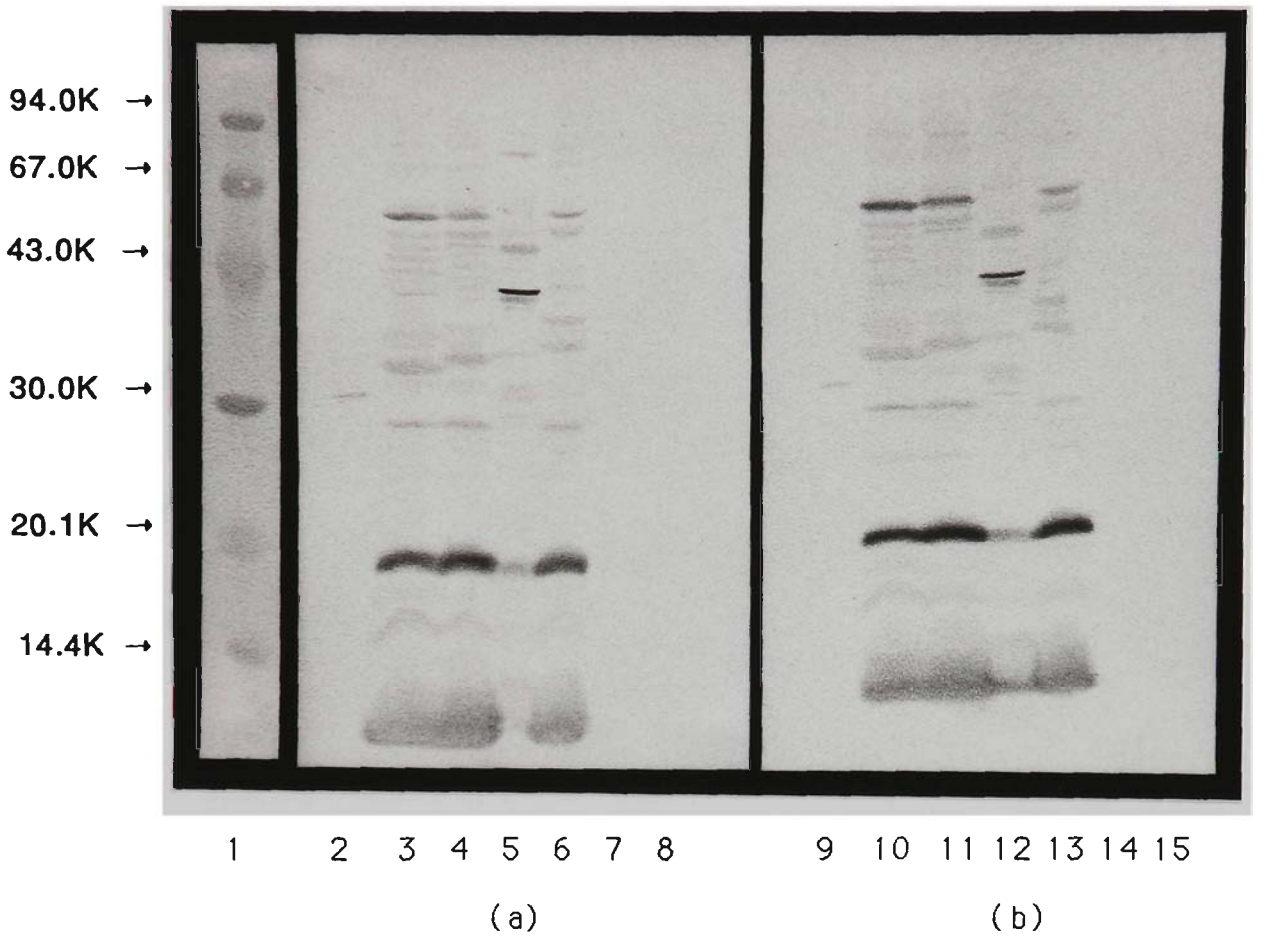


FIGURE 43 - Whole cell protein preparations of laboratory strains reacted with both anti-C and corresponding pre-immune IgG

Western blots reacted with both the anti-C IgG (a) and the pre-immune IgG (b) are illustrated. The serological reaction patterns are presented for B. subtilis (Lanes 2 and 9), E. coli (Lanes 3 and 10), P. aeruginosa (Lanes 4 and 11), P. fluorescens (Lanes 5 and 12), S. typhimurium (Lanes 6 and 13), C. insidiosum (Lanes 7 and 14) and C. michiganense (Lanes 8 and 15). Approximately 25µg whole cell protein was loaded per well. Both anti-C IgG and corresponding pre-immune IgG were used at a concentration of 1.0µg/ml. Lane 1 contains the low molecular weight markers.

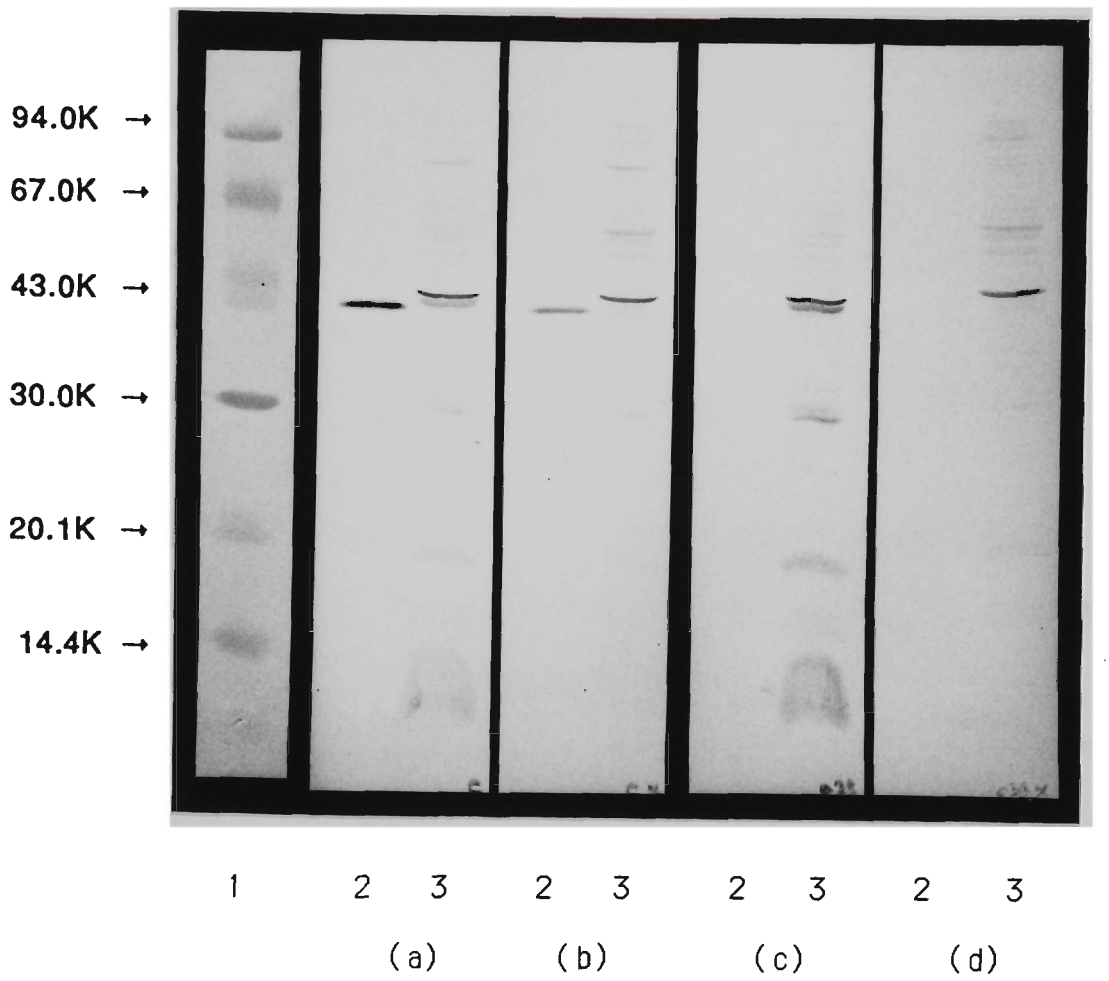


FIGURE 44 - The effect of cross absorbing anti-C IgG with P. fluorescens on the reaction with whole cell preparations of isolate RE01 and P. fluorescens in a western blot

The serological reaction pattern for isolate RE01 and P.fluorescens are illustrated in lanes 2 and 3 respectively. Blot (a) was reacted with anti-C IgG, blot (b) with anti-C IgG cross absorbed with P. fluorescens, blot (c) with the anti-C pre-immune IgG and blot (d) with the anti-C pre-immune IgG cross absorbed with P. fluorescens. In all cases both the anti-C and corresponding pre-immune IgG were used at a concentration of 1.0µg/ml. Cross absorption was performed with whole cells of P. fluorescens at a concentration of 5×10^9 cells/ml. Lane 1 contains the low molecular weight markers.

aeruginosa. The corresponding pre-immune IgG at the same concentration gave patterns of detection identical to those described for the anti-C IgG.

In a checker board analysis, RE01 whole cell suspensions serially diluted from 1x 10^8 cells/ml to 1 x 10^3 cells/ml, 10µl applications, were reacted with both the anti-C IgG and corresponding pre-immune IgG diluted from 10µg/ml to 0.005µg/ml. In both instances the level of detection reached was 1 x 10^5 cells at an IgG concentration of 0.005µg/ml.

Ten-fold dilutions of formaldehyde fixed, formaldehyde fixed and sonicated and formaldehyde fixed, sonicated and split preparations (Section 2.9.6.2) of SA01 and RE01 cultures at an initial concentration of 1 x 10^8 cells/ml were assayed in a slot-blot as described in section 2.9.6.1. There was no difference in the reactions of these preparations, 10µl applications, with either the anti-C IgG (10µg/ml) or corresponding pre-immune IgG at the same concentration (Figure 45).

The effect of cross absorbing the anti-C IgG and corresponding pre-immune IgG with C. michiganense (the only non putative greening/dieback-associated bacterium to react strongly with both the anti-C IgG and corresponding pre-immune IgG in an earlier dot blot) on the detection of the above preparations was investigated. There was no difference between the anti-C and corresponding pre-immune IgG's. The same results were obtained for both the anti-C IgG and corresponding pre-immune IgG cross absorbed with either LPS (Section 2.9.5.3) or guinea pig serum at a concentration of 10µg guinea pig IgG/ml antibody buffer (Section 2.9.5.3).

SA01 and RE01 suspensions, 10µl applications at a concentration of 1 x 10^7 cells/ml, however, did not react in slot-blots with either the purified anti-C IgG or corresponding pre-immune IgG cross absorbed with SA01 (Section 2.9.5.1). Nevertheless, specific cross absorption occurred with the protocols used.

Furthermore, the effect of changing the pH of the buffers used in the slot-blot immunoassay (Section 2.9.6.1) to pH5 and pH9 was investigated. There were no difference for both the anti-C IgG and the corresponding pre-immune IgG which reacted strongly with formaldehyde fixed suspensions, 10µl, of SA01, SA05, RE01, TA01, TA02, C. michiganense and E. coli at a concentration of 1 x 10^8 cells/ml. Weaker reactions occrred with both the IgG preparations with C. insidiosum, P. aeruginosa and S. typhimurium.

Finally, the supernates of the putative greening-associated bacterial isolates grown for 48 hours in incomplete MIG medium and laboratory strains grown in NB at 25°C were reacted with both the anti-C IgG and corresponding pre-immune IgG, both at a concentration of 10µg/ml. A slot-blot immunoassay was performed on the supernates which were prepared by filtering the 48 hour culture through a 0.22µm Millipore filter and spotting 10µl of the filtrate. The anti-C IgG reacted strongly with the supernates of SA03, SA06, SA10, TA02, US04 and P. aeruginosa. Weaker reactions occurred with supernates of SA01, RE01,TA01, C. insidiosum, C. michiganense and P. fluorescens. The pre-immune IgG on the other hand reacted strongly with P. aeruginosa supernate and weakly with SA06, SA10, AU04, C. insidiosum, C. michiganense and P. fluorescens. Only slight indications of colour development occurred for SA01, SA03, RE01, TA01, TA02 and US04 with the pre-immune IgG. This suggests that the protein to which the anti-C IgG was raised is released into the medium and allows the specific reaction of the isolates.

3.10.4 Reaction of SA01 culture supernate with anti-C IgG in western blots

Having observed that the growth medium supernates of SA01, SA03, SA06, SA10, RE01, TA01,TA02 and US04 reacted with the anti-C IgG in a slot-blot immunassay (Section 3.10.3), the supernatants were analysed in a western blot. An

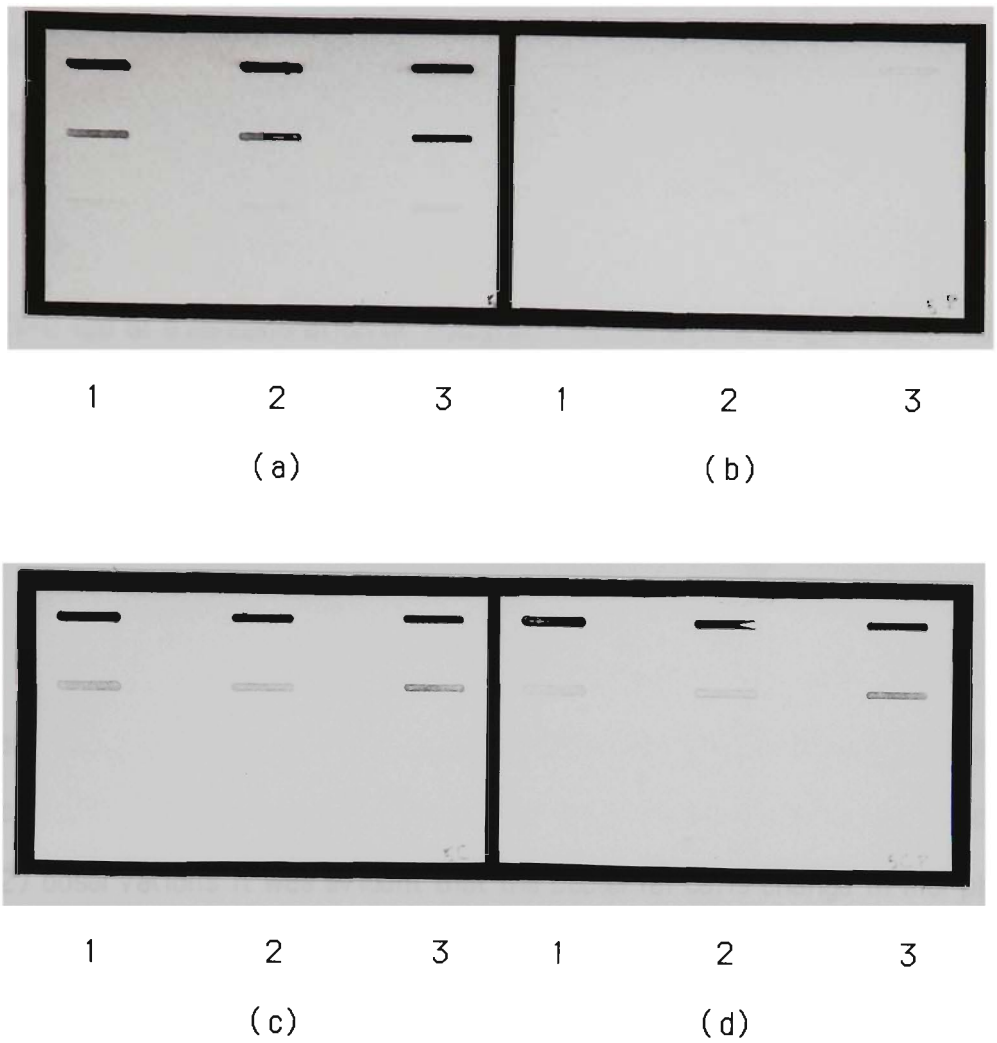


FIGURE 45 - Slot-blot immunoassays comparing the reaction of anti-SA01 IgG and anti-C IgG with differentially treated SA01 suspensions

Ten-fold dilutions (1 x 10^8 cells/ml - 1 x 10^5 cells/ml : applied from top to bottom) of formaldehyde fixed SA01 cells were spotted (Lane 1). Similarly, formaldehyde fixed and sonicated cells were spotted in (2) and formaldehyde fixed, sonicated and split cells were spotted in (3). The blots were reacted with anti-SA01 IgG (a), anti-SA01 pre-immune IgG (b), anti-C IgG (c) and anti-C pre-immune IgG in (d) all at a concentration of 10µg/ml.

overnight culture of SA01 culture grown at 25ºC in incomplete MIG medium was centrifuged at 5,000rpm for 10 minutes at 4ºC. The supernatant was collected and filtered through a 0.22µm Millipore filter. The filtrate (1ml) was freeze dried and resuspended in 100µl PBS and 300µl sample buffer (Section 2.8.1). The bacterial pellet was prepared as described in sections 2.8 and 2.9.7. Approximately 35µg of SA01 whole cell protein and SA01 supernate protein was loaded.

The anti-C IgG at a concentration of 10µg/ml reacted with a single 39K - 40K protein band in both the whole cell and supernate preparations (Figure 46). No bands reacted ted with the corresponding pre-immune IgG at the same concentration.

3.11 Variability in serological reactivity with growth

The many serological observations reported were limited to overnight/exponential phase cultures. From the growth curve (Section 3.2) and plate culture (Section 3.1.2.2) observations it was evident that the bacterial cells change in morphology with age and nutrient availability. Indeed, if the antisera raised, are to be applied to the detection of the putative greening/dieback-associated isolates, then it must be known if the morphological variations of the organism are equally reactive serologically.

Single liquid culture growth curves were performed (Section 2.5) for SA01and TA02 as representative cultures for the SA01, SA03, SA05, SA06, RE01, TA01 and TA02 group of isolates that had similar protein patterns (Section 3.8). The isolates were grown in incomplete MIG medium at 25ºC and the growth monitored by recording the absorbance of 1ml samples taken at regular time intervals, diluted 1/10 and plotted as a function of time (Figure 47). Samples taken at 12, 48, 120, 192 and 288 hours post inoculation, representing the logarithmic, decelerating,

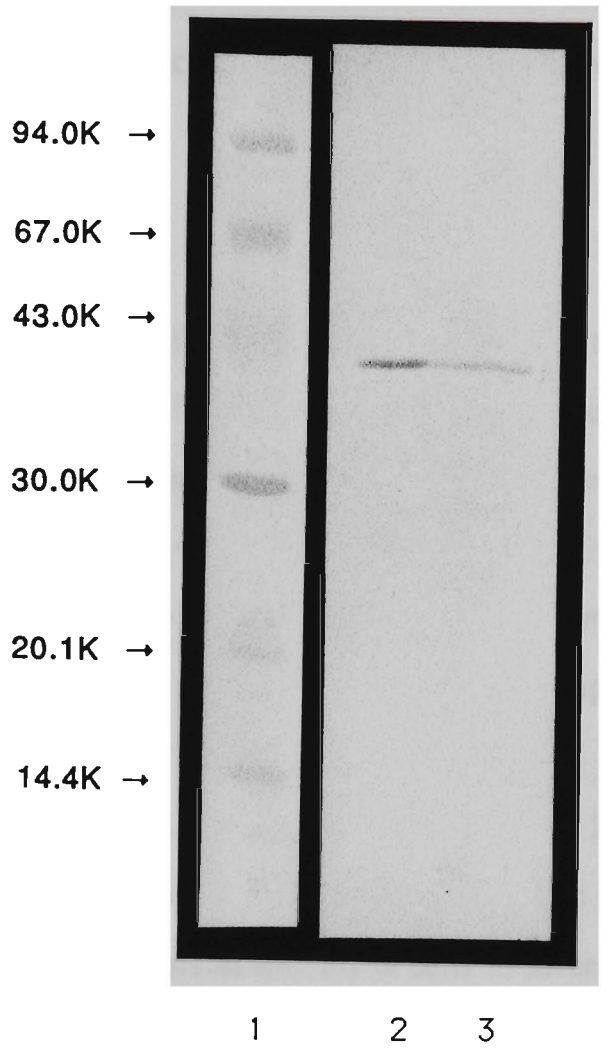


FIGURE 46 – Serological reaction pattern of SA01 whole cell preparation and SA01 culture supernate with anti-C IgG

The serological reactivity patterns for isolate SA01 and corresponding supernatant are presented (Lanes 2 and 3, respectively). Only a single 39K - 40K protein reacted with the anti-C IgG at a concentration of 10µg/ml. Approximately 35µg of both RE01 whole cell sample and of the supernatant sample was loaded. Lane 1 contains the low molecular weight markers.

stationary, late stationary and death phases of growth respectively, were evaluated morphologically and treated for electrophoretic analysis of cell proteins and western blots as described in sections 2.8 and 2.9.7 respectively.

3.11.1 Cell morphology

The morphological changes of cells of isolates SA01 and TA02 during growth (Figure 48) were similar to those described in section 3.2. The 12 hour (log phase) sample contained small thin rods which became shorter, fatter and developing bulbous areas characteristic at 48 hours (decelerating phase). These club-shaped or dumb-bell shaped structures were predominant at120 hours (stationary phase of growth). At 192 hours (late stationary phase) the cells appeared to lose the rod shaped morphology and only the bulbous structures remained. This sample also contained an abundance of lysing cells and cellular debris. At 288 hours (death phase of growth), morphologically distinct cells, whether rod shaped or bulbous, were absent and the culture consisted entirely of cells in several stages of lysis and cellular debris.

3.11.2 Whole cell protein patterns

Protein patterns were established for the 12, 48, 120, 192 and 288 hour samples to determine if there was any variation in the whole cell protein constitution with growth. Approximately 50µg of protein was loaded for each sample. After SDS-PAGE electrophoresis and Coomassie staining, the resulting protein patterns of both SA01 and TA02 were very similar for each phase of growth (Figure 49). There appeared to be no difference in the protein pattern of either SA01 or TA02 during the different phases of growth. However, the intensity of each protein band increased as the culture aged. This may be attributed to the stationary and death phase samples being more soluble than the log and decelerating phase samples even

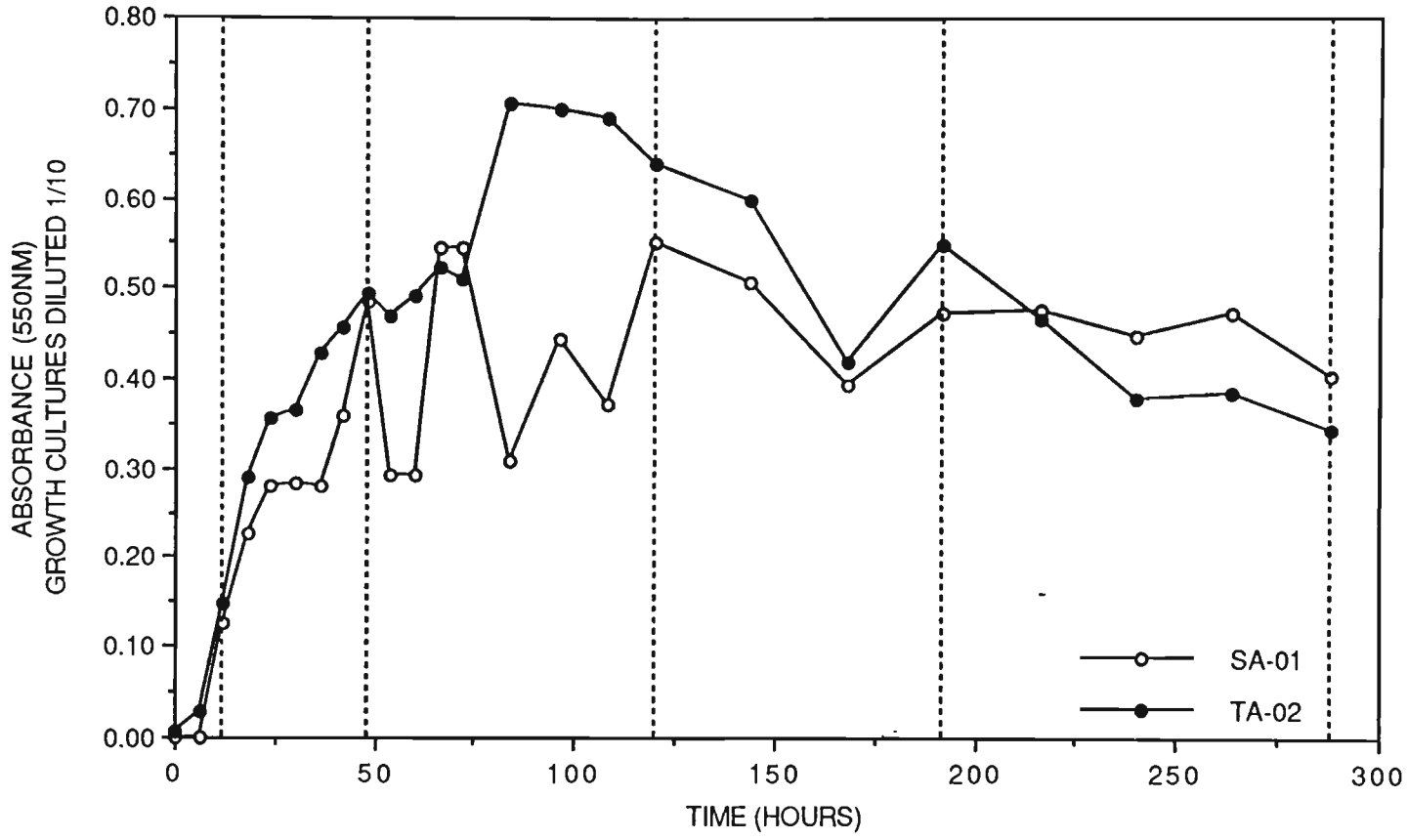


FIGURE 47 - 25°C culture growth curves for isolates SA01 and TA02

The absorbance at 550nm (1cm light path) of samples diluted 1/10 were plotted as a function of time. (---) represents the times at which samples were taken for electrophoretic analyses of whole cell proteins and western blots.

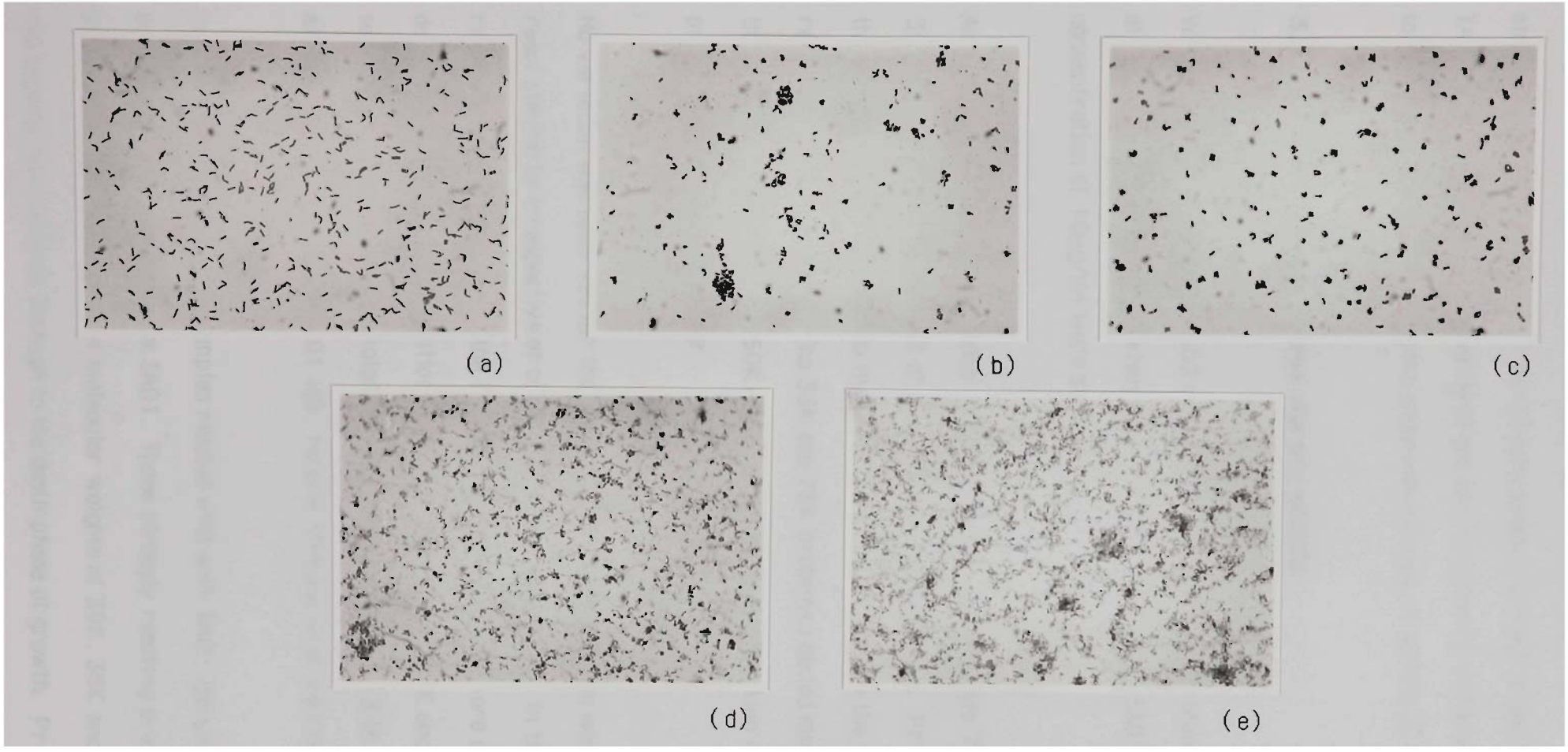


FIGURE 48 – Gram stains of isolate SA01 cells during growth at 25°C

Micrographs of stained smears showing the cell morphology at 12 hours (a), 48 hours (b), 120 hours (c), 192 hours (d) and 288 hours (e) during growth in liquid culture of isolate SA01 at 25°C. (Magnification: 800x). Similar observations were made for isolate TA02.

though the overall protein concentration was standardized. Not only was the similarity of the protein patterns for each phase of growth evident in both SA01 and TA02 but so too was the similarity of the culture patterns of the bacterial cultures to one another complementing the observations made in section 3.7.

3.11.3 Antigenic variation during growth

Western blots of SA01 and TA02 whole cell protein (75µg protein/well) patterns at different stages of growth when reacted with purified anti-SA01 IgG at a concentration of 10µg/ml were similar (Figure 50).

Anti-SA01 reacted with proteins of apparent molecular weights 77K, 72K, 60K, 39K, 35K, 34K, 23K and 16K of a SA01 pattern in log phase. Proceeding through the decelerating phase and into the stationary phase of growth the serological reactivity pattern changed. The 35K and 23K proteins reacted more strongly with the anti-SA01 IgG while the 60K reacted weakly and the reaction with a 16K protein disappeared altogether.

No variation was observed for the 77K, 72K and 39K proteins which appeared to react similarly irrespective of culture age. A marked change in the serological reaction pattern occurred in the late stationary and became more evident in the death phase of growth. In addition to the 77K, 72K, 39K, 35K and 23K proteins, several proteins ranging in molecular weights from 103K - 133K and 19K - 22K also reacted with the anti-SA01 IgG. No pre-immune observations were available.

Isolate TA02 growth phase samples reacted with anti-SA01 IgG gave a similar serological reaction pattern as SA01. Three strongly reacting proteins in the log phase of growth with apparent molecular weights of 39K, 35K and 23K persisted and become more intense through to the death phase of growth. Proteins with

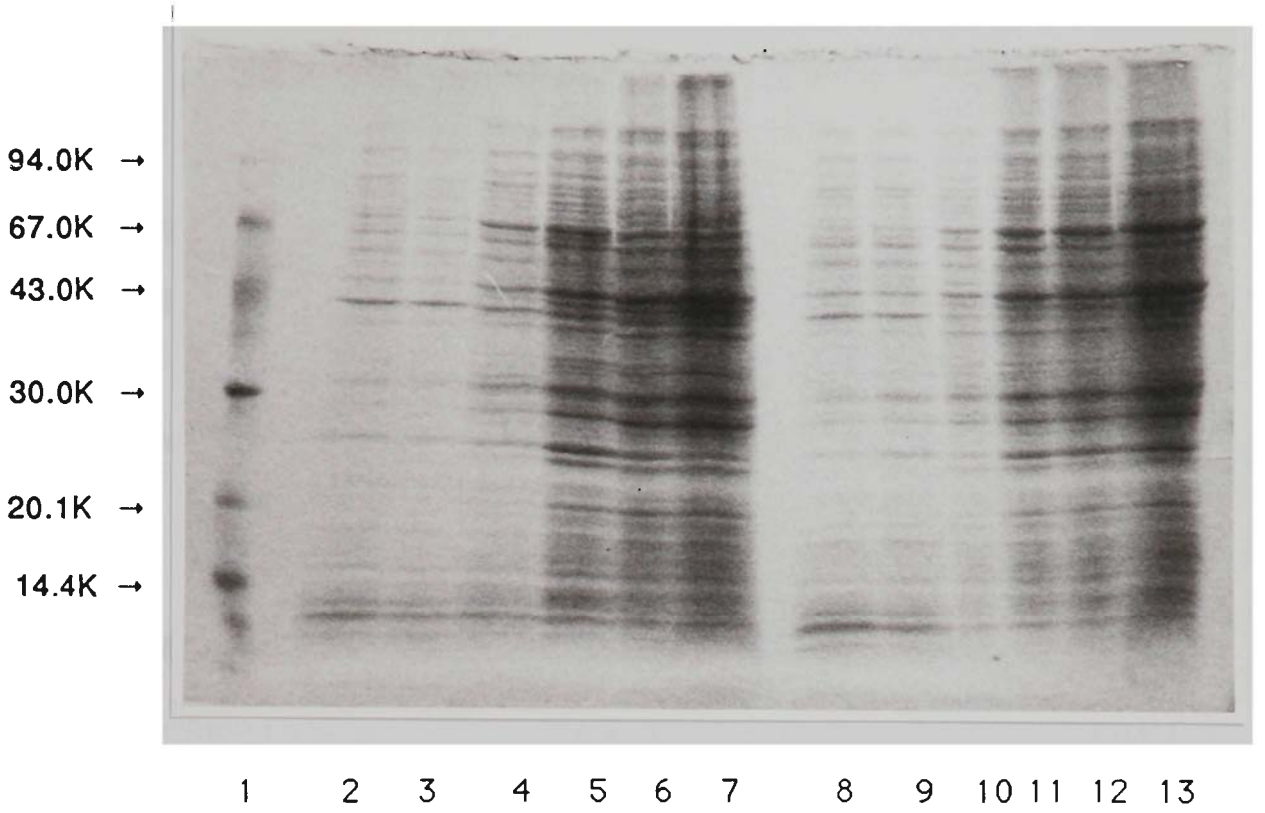


FIGURE 49 - Whole cell protein patterns at different stages of growth

SA01 and TA02 whole cell protein samples taken at different times during growth in liquid culture at 25°C were analysed by SDS-PAGE. The patterns obtained from 50µg of protein loaded for each sample are illustrated. SA01 and TA02 patterns are represented by lanes 2 - 7 and 8 - 13, respectively. Lanes 2 and 8 represent the protein patterns of overnight cultures. Lanes 3 and 9 are the protein patterns for the 12 hour (log phase samples); lanes 4 and 10 represent the 48 hour (decelerating phase samples); lanes 5 and 11 represent the 120 hour (stationary phase samples); lanes 6 and 12 represent the 192 hour (late stationary phase samples) and lanes 7 and 13 represent the 288 hour (death phase samples). Lane 1 contains the low molecular weight markers.

apparent molecular weights of 77K, 72K and 16K present in the log phase culture, decreased in intensity with growth. The serological reaction with a 16K protein disappeared altogether. During the stationary phase, proteins with apparent molecular weights of 121K, 114K and 79K appeared and persisted until the death phase.

The serological reaction patterns in western blots using anti-SA01 IgG reacted with whole cell protein preparations for isolates SA01 and TA02 during growth are very similar. Of particular interest are the 77K, 72K, 39K and 23K proteins that reacted with the anti-SA01 IgG in all the phases of growth of both SA01 and TA02 isolates.

3.12 Applications to affected and healthy citrus tissue

3.12.1 Reaction of greening/dieback affected citrus preparations with anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG in slot-blot immunoassays

Slot-blot immunoassays have been performed on greening affected citrus tissue preparations with anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG's. No reactions with the corresponding pre-immune IgG were observed (H.M. Garnett, personal communication). A precaution that has to be taken when screening plant tissue is that the IgG to be used must be cross absorbed with healthy tissue so as to prevent any non specific binding resulting in false positive reactions. Possible false positive reactions caused by other infections are, however, not eliminated in this way. Some antisera readily bind non specifically to plant tissue in slot-blot immunoassays if they have not been cross absorbed.

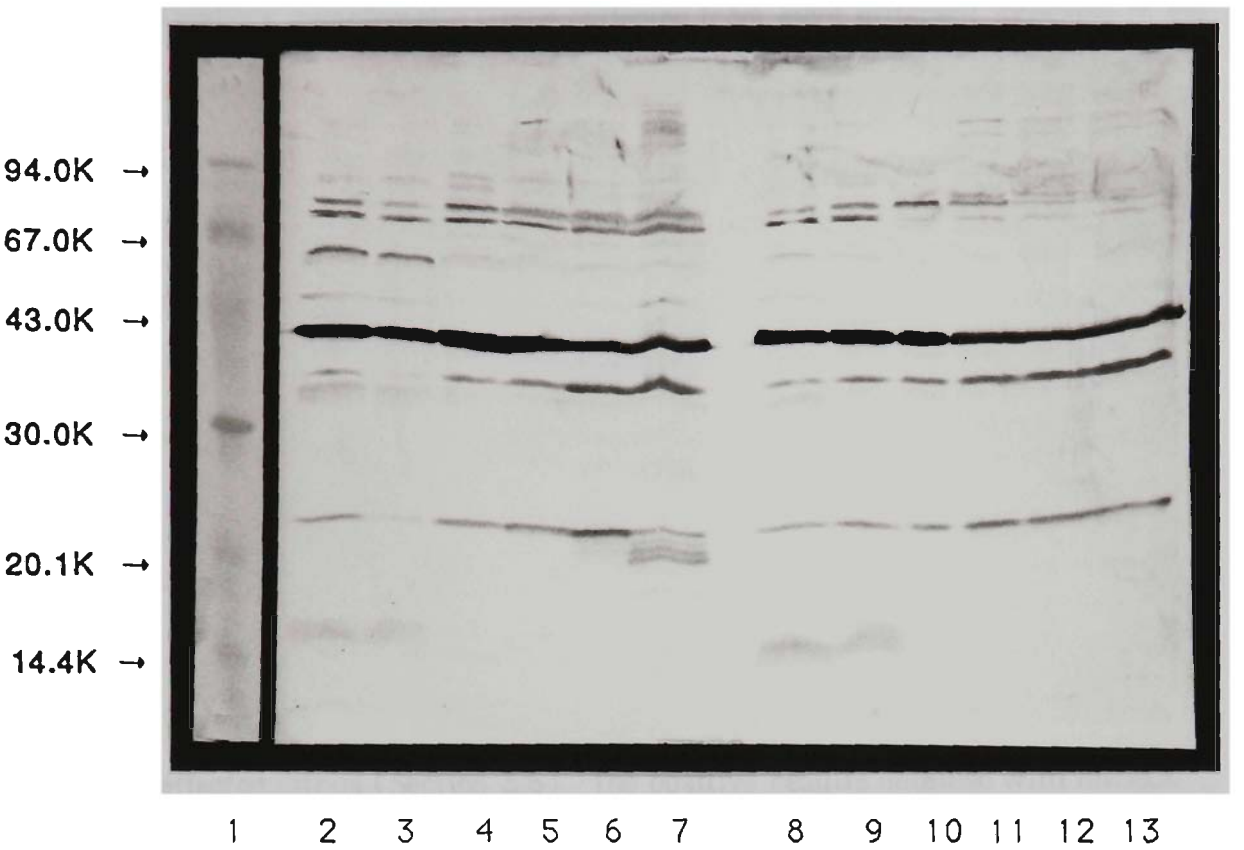


FIGURE 50 - Western blot analysis with anti-SA01 IgG of whole cell protein preparations during growth of isolates SA01 and TA02

Serological reactions of SA01 and TA02 whole cell proteins during growth in liquid culture at 25°C, following electrophoretic separation of 75µg protein and subsequent reaction with anti-SA01 (10µg/ml) in a western blot, are illustrated by lanes 2 - 7 and 8 - 13 respectively. Lanes 2 and 8 represent the patterns of overnight cultures. Lanes 3 and 9 are the patterns for the 12 hour (log phase samples); lanes 4 and 10 represent the 48 hour (decelerating phase samples); lanes 5 and 11 represent the 120 hour (stationary phase samples); lanes 6 and 12 represent the 192 hour (late stationary phase samples) and lanes 7 and 13 represent the 288 hour (death phase samples). Lane 1 contains the low molecular weight markers.

Australian dieback symptomatic tissue collected from eight different locations in the MIA were screened in a slot-blot immunoassay (Section 2.9.6.3) with anti-SA01, anti-SA03 and anti-RE01 IgG at a concentration of 10µg/ml and cross absorbed with healthy grapefruit tissue as described in section 2.9.5.2 (Figure 51). Anti-SA01 IgG reacted with three samples. Anti-SA03 reacted with six of the samples tested while anti-RE01 reacted with four of the samples. In each case no positive reactions were observed for the non symptomatic tissue collected from the same areas and presumed to be healthy. No reactions occurred with the corresponding pre-immune IgG.

The IgG's used were raised against bacterial isolates from African and Reunion greening affected citrus (Section 3.5). The positive results obtained with dieback affected citrus using these IgG's consequently suggest that there is a common factor relating Australian dieback to African and Reunion greening. Furthermore, the potential use of these antisera to detect dieback may be viable.

3.12.2 Effects of cross-absorbing the antisera with healthy citrus tissue

The antisera were raised not only to identify the greening organism in culture but also apply the detection systems established in vitro to in vivo conditions, that is, to detect the presumptive greening-associated bacterial pathogen in diseased plant tissue. Consequently, the effects of cross absorbing the antisera with healthy plant material, as described in section 2.9.5.2, on the recognition with bacterial cell proteins were determined.

The patterns observed for SA01, SA03 and RE01 in western blots prepared as described in section 2.9.7 reacted with the corresponding anti-SA01, anti-SA03 and anti-RE01 IgG (Figure 52), were almost identical to those reacted with anti-

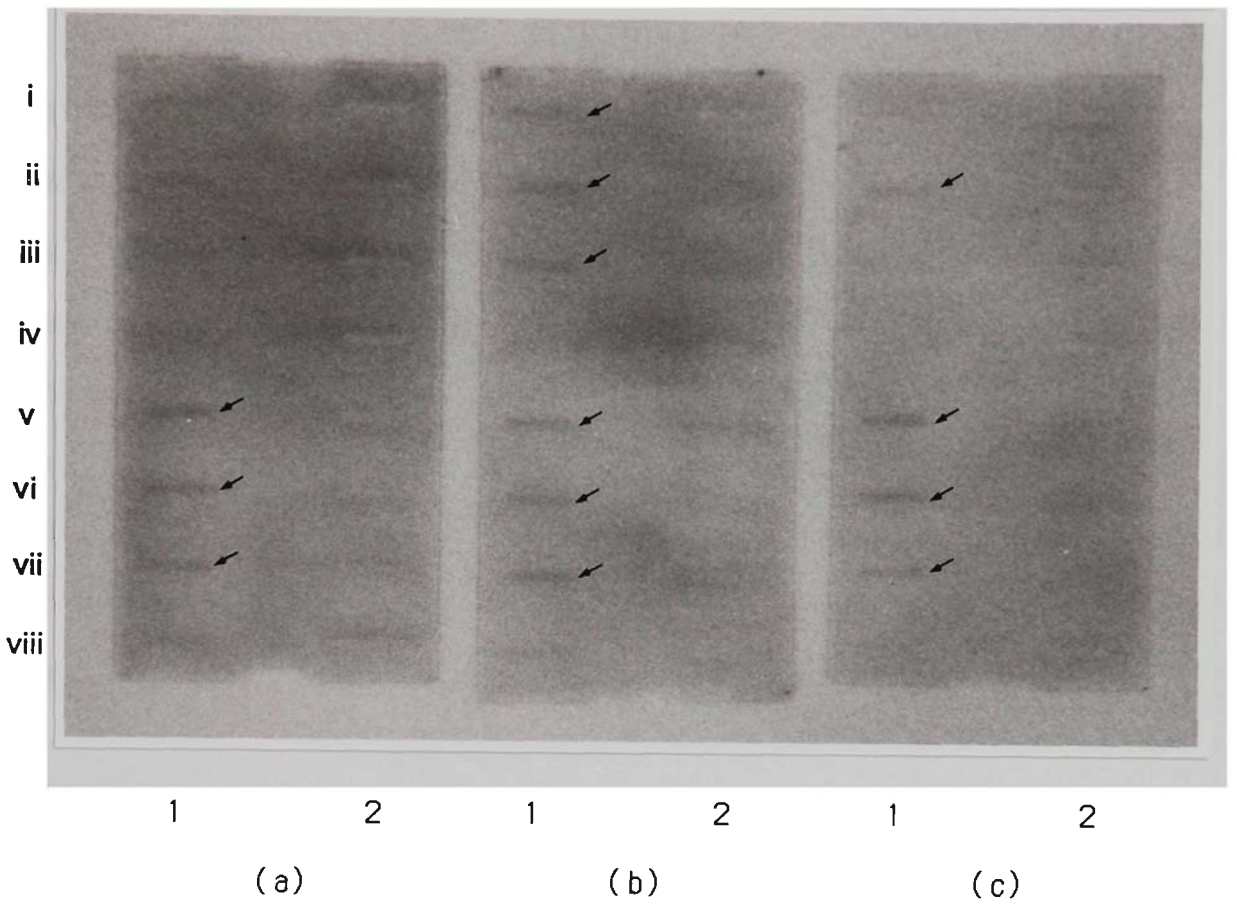


FIGURE 51 - Slot-blot immunoassay of Australian dieback affected tissue

An autoradiograph of a slot-blot immunoassay of preparations from affected dieback and non symptomatic leaf tissue collected from different areas in the MIA screened with SA01, SA03 and RE01 IgG (10µg/ml) cross absorbed with healthy grapefruit is illustrated. Blots (a), (b) and (c) represent the reactions for anti-SA01 IgG, anti-SA03 IgG and anti-RE01 IgG respectively. Lanes 1 and 2 represent the dieback affected tissue and the non symptomatic healthy controls in each case. Rows i - vii represent different locations from which the samples were collected. Arrows indicate positive reactions.

SA01, anti-SA03 and anti-RE01 IgG cross absorbed with healthy grapefruit tissue. In both cases, the concentration of IgG was 10µg/ml. Only in SA03 was the reaction with a single 65K - 66K protein eliminated with healthy grapefruit tissue.

Similarly, the reaction of the 38K - 40K protein band was not affected by cross absorbing the anti-C IgG at a concentration of 10µg/ml with healthy grapefruit tissue (Figure 53). During the affinity purification of the IgG fraction and the associated concentration steps however, the anti-C IgG acquired the ability to react with a protein of apparent molecular weight 64K - 65K and a group of proteins in the 25K molecular weight range of a RE01 protein pattern. The reaction of the anti-C IgG to the diffuse protein band in the 25K region was, however, eliminated by absorption with healthy grapefruit while the reaction with the 64K - 65K protein was unaffected by cross absorption.

It appears, therefore, that the anti-SA01, anti-SA03, anti-RE01 and anti-C IgG's maintain their overall reactivity with the several bacterial proteins in the presence of plant tissue.

The <u>in vitro</u> observations have suggested that the purified anti-SA01, anti-SA03, anti-SA07, and anti-RE01 IgG's react with the putative greening/dieback-associated isolates in slot-blot immunoassays and western blots, and anti-C IgG reacts with proteins in western blots. Indeed, the antisera have already successfully been applied in the detection of African and Asian greening affected plant tissue (H.M. Garnett, personal communication) and Australian affected dieback material (Section 3.12.1) in slot-blot immunoassays. Investigations were consequently carried out to determine if the western blot techniques developed for the putative greening/dieback isolates in culture could be applied to affected plant material.

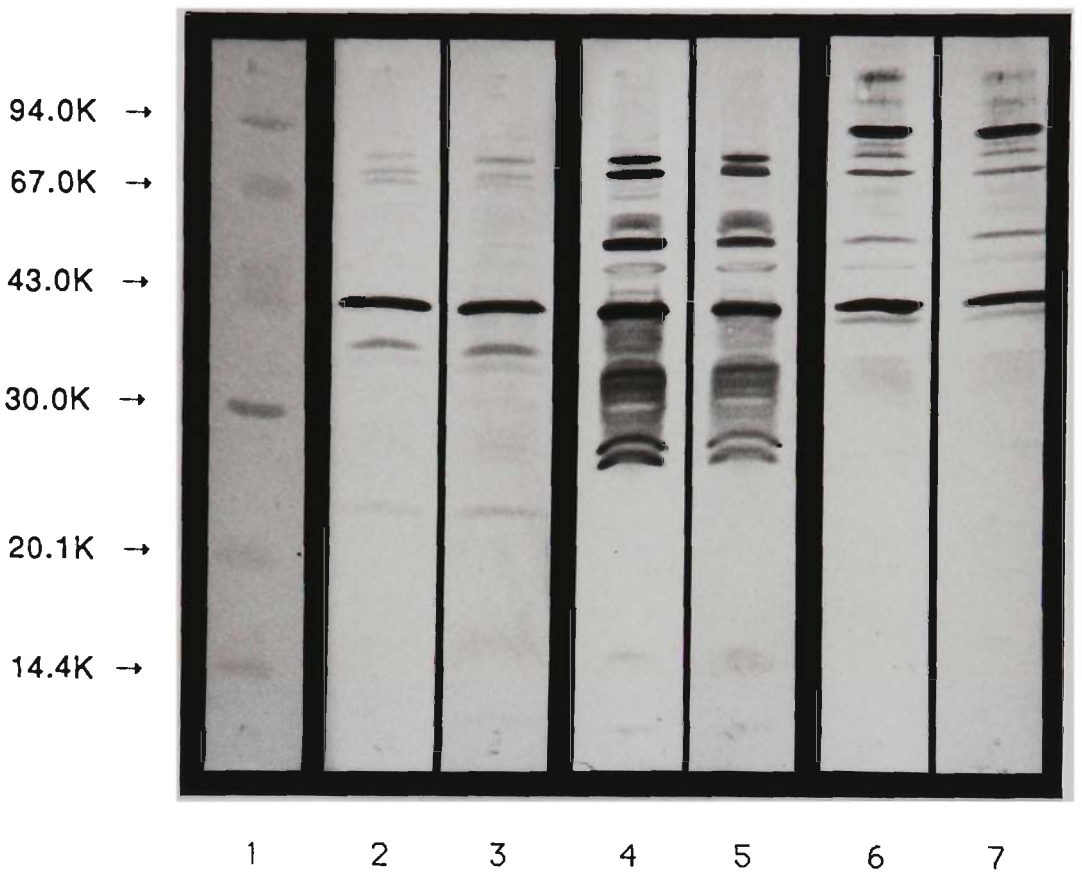


FIGURE 52 - Effects of cross-absorbing anti-SA01, anti-SA03 and anti-RE01 IgG

Western blots of whole cell protein preparations from isolates SA01 (Lanes 2 and 3), SA03 (Lanes 4 and 5) and RE01 (Lanes 6 and 7). The effect of cross-absorbing anti-SA01, anti-SA03 and anti-RE01 with healthy plant material on the reactions of SA01, SA03 and RE01 proteins is illustrated (lanes 3, 5 and 7, respectively). Compared to the non cross-absorbed IgG preparations (Lanes 2, 4 and 6), there was no detectable effect. Only the reaction of a single SA03 64K - 65K protein with the anti-SA03 IgG was eliminated by absorption with plant tissue. Approximately 75µg protein/well was loaded. Both the cross-absorbed and non cross-absorbed IgG's were used at 10µg/ml. Lane 1 contains the low molecular weight markers.

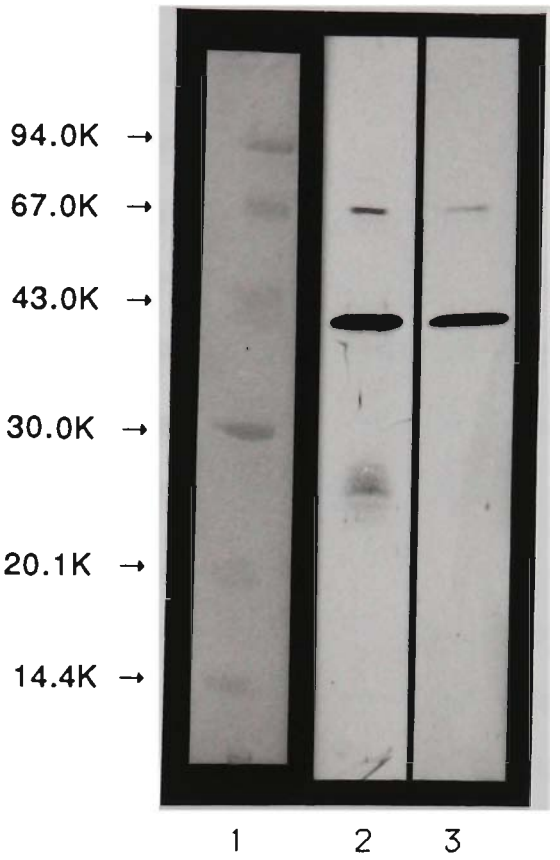


FIGURE 53 - Effect of cross-absorbing anti-C IgG

The effect of cross absorbing anti-C IgG at a concentration of 10µg/ml with healthy grapefruit tissue on the reaction with RE01 whole cell protein preparations (50µg of protein was loaded) is illustrated (Lane 3). Lane 2 represents a duplicate western blot of RE01 whole cell proteins reacted with non cross absorbed IgG at the same concentration. The reaction of the 37K - 38K protein is not affected while the intensity of reaction is decreased for the 64K - 65K protein and eliminated for the protein bands around the 25K molecular weight range. Lane 1 contains the low molecular weight markers.

3.12.3 Evaluation of preparations from affected field samples reacted with anti-C IgG in western blots

Both greening affected and dieback affected field samples were accordingly prepared for reaction in western blots. Infected and healthy midribs were homogenized 1:10 (w/v) in PBS and prepared for electrophoresis as described in section 2.9.6.4. Controls included healthy grapefruit tissue homogenized 1:10 (w/v) in PBS containing 1×10^8 and 1×10^5 REO1 cells/ml and further treated as described for the affected and healthy plant tissue samples. SDS-PAGE was performed (Section 2.8.2) and the resulting patterns blotted (Section 2.9.7).

The western blots were reacted with both anti-C IgG at a concentration of 10µg/ml and the corresponding pre-immune IgG at the same concentration, both of which had been cross absorbed with healthy grapefruit tissue as described in section 2.9.5.2. The antibody-antigen reactions were detected using ^{125}I conjugated second antibody diluted 1/500 (Section 2.9.6.3).

After 21 days at -80°C, the autoradiographs were developed and although traces of the lanes where the samples had run could be discerned, no antigenic bands were detected. Furthermore, no antigenic bands were detected in the spiked samples suggesting the concentration of the antigen was too low for visible reaction. Consequently, healthy plant material was spiked with ten-fold dilutions of whole bacterial cell preparations and the minimum level of visible reaction with the purified IgG's in western blots determined.

3.12.4 Reactions of whole cell antigens of isolate RE01 in spiked healthy grapefruit tissue preparations

A pellet of isolate RE01 containing 1 x 10^{10} cells was resuspended in a homogenized healthy Marsh grapefruit midrib preparation (1:5 w/v PBS) and serially diluted to 1 x 10^{3} cells/ml in the plant homogenate. Controls included a suspension of isolate RE01 cells standardized to an absorbance of 0.5 at 550nm (approximately 2 x 10^{9} cells/ml) and unspiked plant homogenate. The samples were further prepared as described in section 2.8 and treated as described in section 2.9.7. The resulting equivalent cell concentration in the spiked samples loaded for SDS-PAGE was 2.5 x 10^{9} - 2.5 x 10^{3} cells/100µl applied.

The characteristic western blot serological reactivity pattern for isolate RE01 control occurred when reacted with anti-RE01 IgG at 10µg/ml (Figure 54). The same pattern also occurred in the spiked plant samples containing the 2.5 x 10^{9} and 2.5 x10^{8} cells/well. A marked decrease in the intensity of the serological reaction pattern occured in the plant sample spiked with 2.5 x 10^{7} cells/well. Only a single protein band of molecular weight 38K - 40K was evident for the sample containing the equivalent of 2.5 x 10^{7} cells/well. Although the blot (Figure 54) suggests the detectable reaction level as 1,000-fold more sensitive, it is not possible to establish with certainty that the protein band observed for the lane loaded with 2.5 x 10^{4} cells/well is not a run off from the adjacent lanes. It is indeed likely that this is the case. No plant protein cross reactions were detected by the anti-RE01 IgG in the unspiked healthy tissue. The pre-immune IgG (10µg/ml) did not react with any of the bacterial proteins observed.

A modification to the above technique adopting a more dilute plant preparation and spiked with fewer bacteria was investigated. Healthy Sexton tangerine midribs

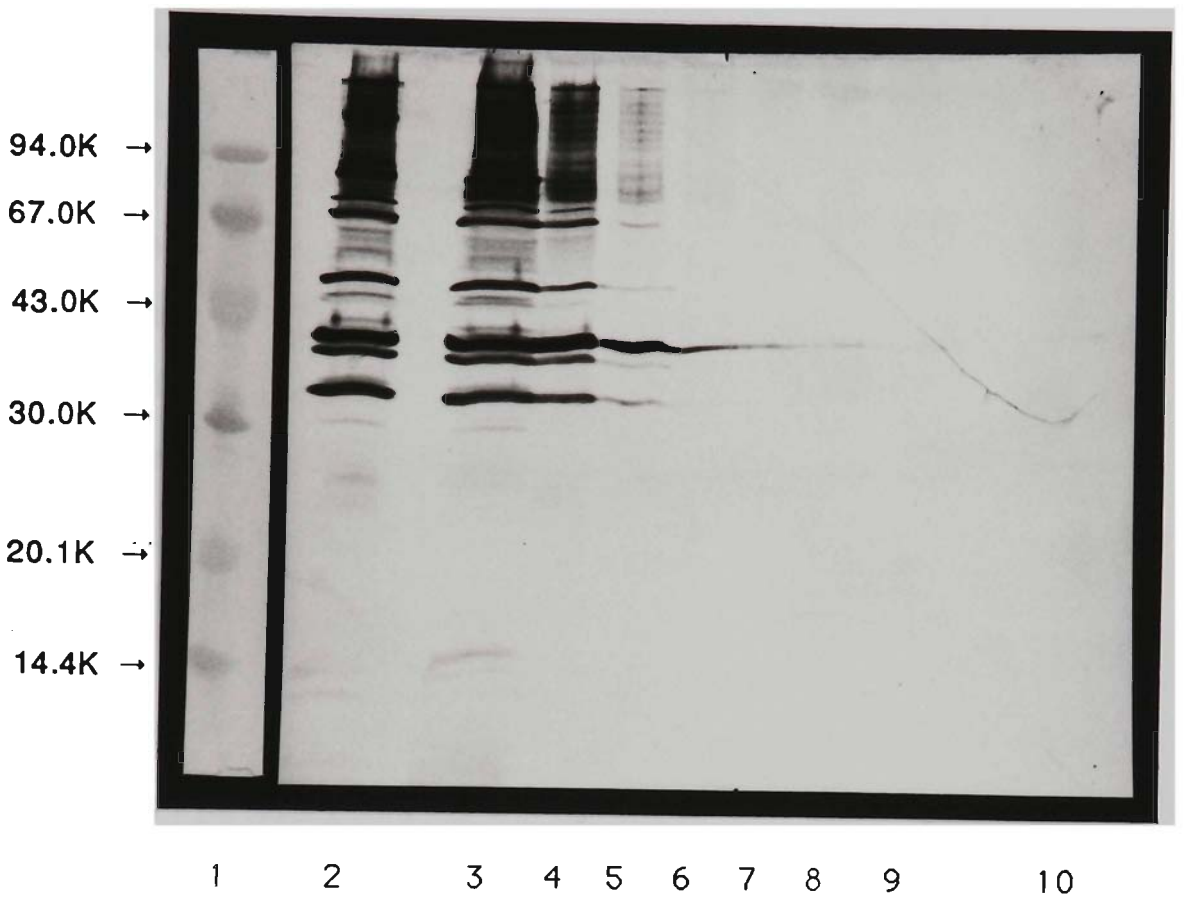


FIGURE 54 - Serological reaction patterns of plant tissue preparations spiked with REO1 and reacted with anti-REO1 IgG

The western blot illustrates the serological reaction patterns of plant tissue preparations spiked with various levels of whole bacterial cells of isolate REO1 and reacted with anti-REO1 IgG at a concentration of 10mg/ml. Lane 2 and 10 represent the patterns for isolate REO1 and healthy plant tissue controls, respectively. Lanes 3 to 9 contain the spiked plant extracts. The bacterial cell number decreases ten-fold from 2.5×10^9 in lanes 2 and 3 to 2.5×10^3 cells in lane 9. The amount of plant material remains constant for all the samples. Lane 1 contains the low molecular weight markers. There was no reaction with the corresponding pre-immune IgG at the same concentration.

homogenized 1:10 (w/v PBS) were filtered through a single layer of Miracloth® (Chicopee Mills Inc., Milltown, NJ, U.S.A.). An overnight culture of isolate SA01 grown in incomplete MIG medium at 25°C was adjusted to a concentration of absorbance 0.5 at 550nm (approximately 1 x 10^9 cells/ml), centrifuged and resuspended in the plant preparation. Ten-fold dilutions of the plant/SA01 suspension in 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV), as well as isolate SA01 (standardized as described) and an unspiked plant control were also prepared. All the samples were sonicated for 1 hour in a Bransonic B-12 sonicating ice bath (Branson cleaning equipment company, Shelton, Conn, U.S.A.) and prepared for electrophoresis and western blots as described in sections 2.8 and 2.9.7 respectively.

Western blots were developed using anti-C IgG at a concentration of 10µg/ml. Two protein bands with apparent molecular weights of 39K and 28K (Figure 55) were observed in the lane containing the SA01 control preparation. Both these bands were also observed in the spiked tissue preparations in the lanes containing 1.25 x 10^8 and 1.25 x 10^7 cells/well. The 39K protein was just visible in the lane containing 1.25 x 10^6 cells/well while the 28K protein was evident in the lane containing plant material spiked with 1.25 x 10^3 cells/well. Only the 28K protein was evident in the plant control. This 28K protein also reacted with the corresponding pre-immune IgG (10µg/ml) and appears to be a plant protein.

Both an alkaline phosphatase conjugated and ^{125}I conjugated second antibody were tried. There was no apparent difference in the level of sensitivity of either antibody. In both cases approximately 1.25 x 10^9 - 1.25 x 10^7 cells/well could be detected.

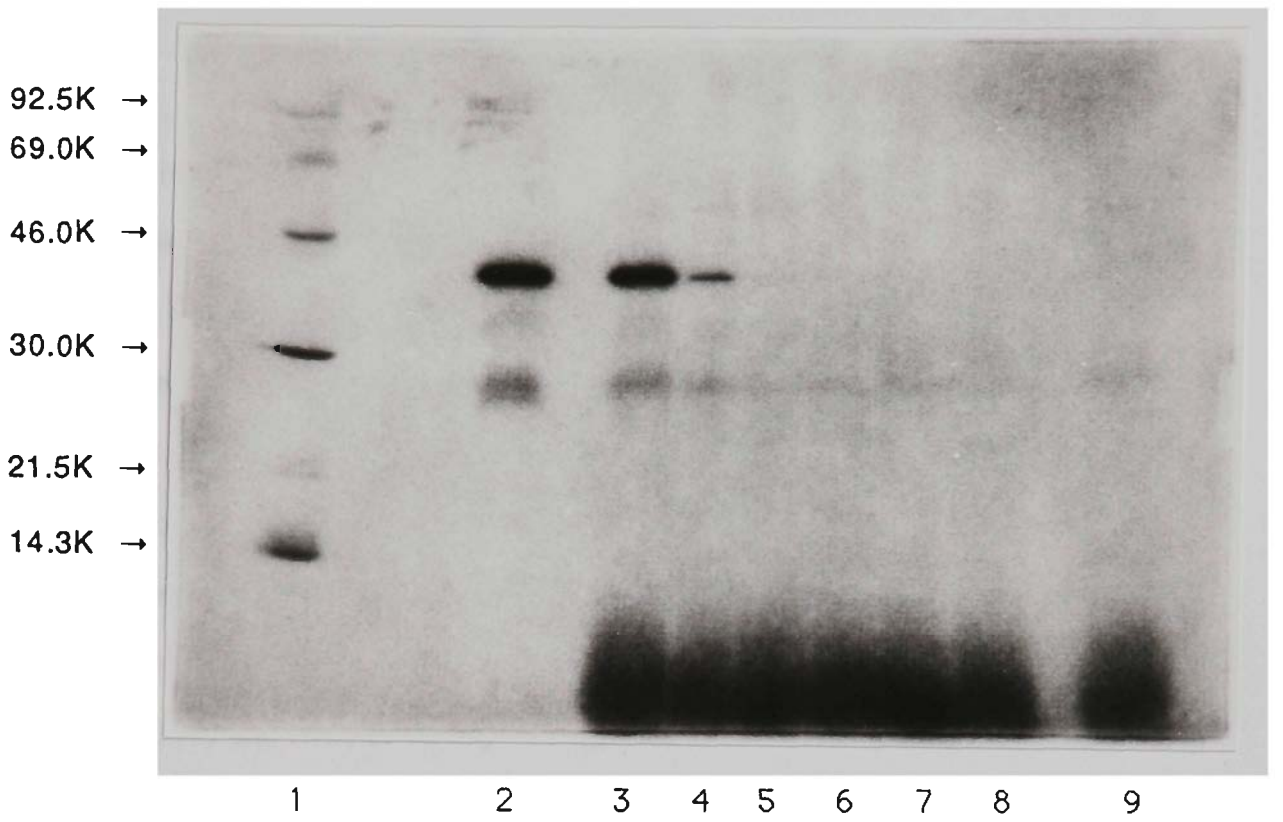


FIGURE 55 - Serological reaction patterns of plant preparations spiked with SA01 and reacted with anti-C IgG

An autoradiograph of plant material spiked with ten-fold serial dilutions of isolate SA01 and reacted with anti-C IgG (10µg/ml) is illustrated. A protein with an apparent molecular weight of 39K was reactive in the bacterial control (Lane 2) and in the spiked plant material containing 1.25 x 10^9 cells/well (Lane 3) - 1.25 x 10^7 cells/well (Lane 5). This protein did not react detectable in the plant control (Lane 9) or by the corresponding pre-immune IgG at the same concentration. The 27K - 28K protein observed in lanes 2 through 9 was also reacted with the corresponding pre-immune IgG. An identical pattern was obtained for the GAR-AP conjugated second antibody system. Lane 1 contains the low molecular weight markers.

3.12.5 Serological reactions of antigens in healthy grapefruit tissue spiked with isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 culture supernates with anti-C IgG in western blots

Only limited volumes of each of the SA01, SA03, SA05, SA06, RE01, TA01 and TA02 culture supernates previously used in the slot-blot immunoassay (Section 3.5.2.2) were available at the time. Consequently these were pooled and filtered through a 0.22µm Millipore filter. Healthy grapefruit leaf midribs were homogenized 1:10 (w/v) in 0.15M phosphate buffered saline (PBS) pH 7.2 (Appendix IV) and diluted 1:1 with two-fold dilutions (1/1 - 1/16) of the supernate preparation in PBS. These suspensions were sonicated for 1 hour in a Bransonic B-12 sonicating ice bath (Branson cleaning equipment company, Shelton, Conn, U.S.A.) and filtered through a single layer of Miracloth® (Chicopee Mills Inc., Milltown, NJ, U.S.A.). Each filtrate (approximately 3ml), 1.5 ml of the originally filtered culture supernate and 1.5 ml of the plant homogenate was freeze dried and resuspended in 50µl PBS and 150µl sample buffer (Section 2.8). Gels were loaded with 25µl of each preparation and treated as described in sections 2.8 and 2.9.7.

Two protein bands with apparent molecular weights of 38K and 36K reacted with the anti-C IgG at 10µg/ml (Figure 56). Both proteins were evident in the lane containing the original supernate and in the lanes containing the spiked plant material in which the supernate had been diluted 1/1, 1/2, 1/4, 1/8 and 1/16. The protein bands were not apparent in the plant preparation nor did they react with the corresponding pre-immune IgG at the same concentration. The anti-C IgG raised to a single 38K - 40K protein band is in this case noted to react with two bands. The samples loaded were extremely viscous and took a long time to penetrate the stacking gel. From previous western blot serological reaction patterns (Section 3.8), the protein band in the 38K - 40K region was comparatively large and

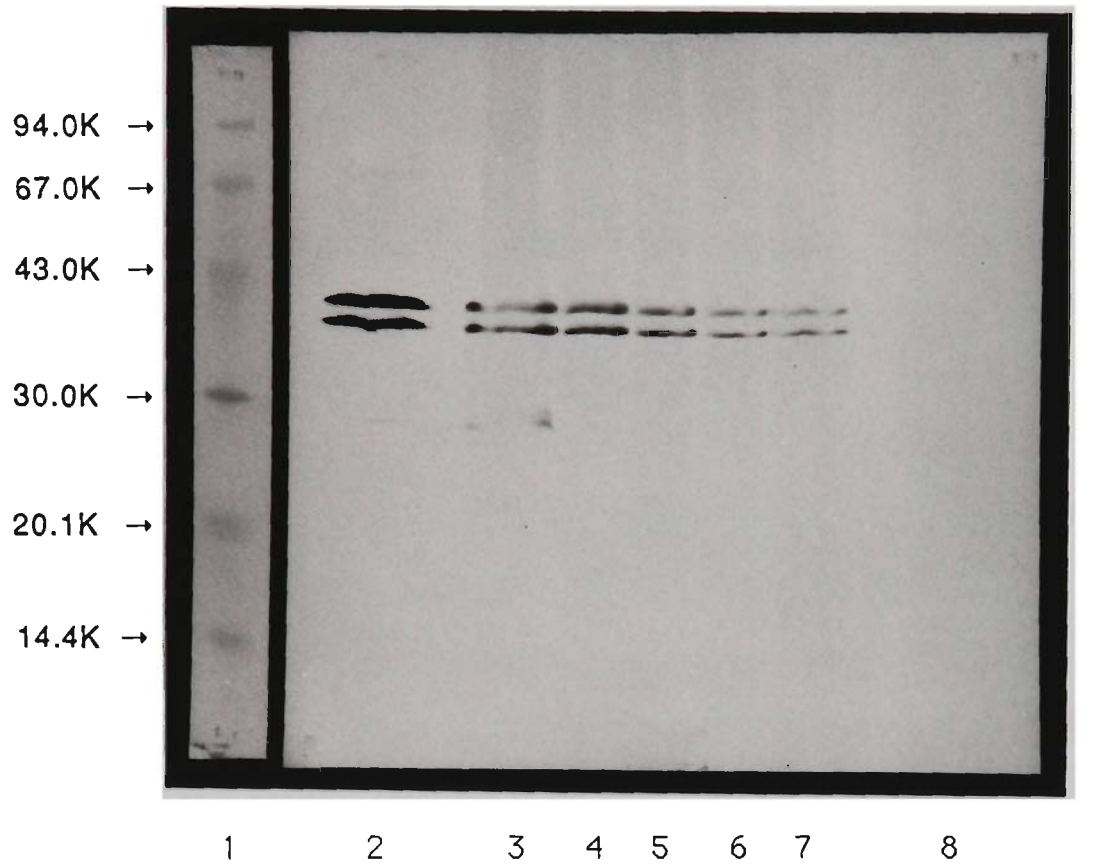


FIGURE 56 - Serological reaction patterns for plant preparations spiked with culture supernates and reacted with anti-C IgG in western blots

A western blot of plant tissue preparations spiked with culture supernates and reacted with anti-C IgG (10µg/ml) is illustrated. Two protein bands with apparent molecular weights of 38K and 36K are evident in both the supernate (Lane 2) and spiked plant samples. The plant samples were spiked with two-fold dilutions (1/1 to 1/16) of the culture supernate (Lanes 3 to 7 respectively). Non specifc binding of the antiserum is not evident in the plant tissue control (Lane 8). There was no corresponding pre-immune IgG cross reaction with the 38K and 36K protein bands. Pre-immune IgG was used at a concentration of 10µg/ml. Lane 1 contains the low molecular weight markers.

appeared to have more than one protein occupying a similar position on the blot. The change in the physical conditions of this system may have resolved these proteins.

3.12.6 Serological reaction of proteins in phloem exudates from affected field samples with anti-SA03 IgG and anti-C IgG in western blots

By modifying an assay procedure used to detect blight proteins in citrus roots (R.F. Lee, personal communication) described in section 2.9.6.4, western blots were performed with anti-SA03 IgG and anti-C IgG on phloem exudates taken from dieback affected grapefruit samples collected in three orchards in Griffith (Section 3.4) and which gave positive reactions in dot spots (Section 3.12.1).

Very faint protein bands that reacted with anti-SA03 IgG in the 50K and 30K molecular weight range and bands in the 50K molecular weight range with anti-C IgG were apparent. Both IgG's were used at a concentration of 10µg/ml. Unfortunately owing to the unavailability of more samples and limited time, this experiment could not be repeated. However, proteins in western blots of affected plant tissue preparations do react. Refinement of the procedure may improve the detectable serological reactions. There were no apparent reactions with the corresponding pre-immune IgG or with phloem exudates from non symptomatic plant material collected in the same orchards.

The very low intensity of the reactions of protein bands may suggest a reason why no bands were apparent earlier (Section 3.12.3). Furthermore, the above mentioned method eliminates the presence of plant homogenates that may interfere with the detection of the purified IgG's. This is especially significant when plant extracts are concentrated in an attempt to increase the bacterial concentrations to detectable

levels. The more concentrated samples were poorly resolved in the gels and consequently unsuitable for blotting. In addition, the phloem exudates obtained using the extraction procedure described above are more easily concentrated for reaction in either slot-blot immunoassays or western blots.

4.0 DISCUSSION

There are many symptoms associated with greening-affected citrus (Mc Clean and Oberholzer, 1965; Mc Clean and Schwarz, 1970; Fraser et al.,1966). Indeed, the range of symptoms by which greening is identified have resulted in the description of the same disease by a variety of names. Symptoms of Australian citrus dieback (ACD) affected plants are comparable to those described for greening (Broadbent, 1977[1+2]) in that the trees are sectorially affected, premature leaf fall is evident and multiple buds occur on the defoliated branches. The leaf symptoms resemble zinc deficiency. The characteristic blotchy-mottle also occurs. Some leaves have a yellowing midrib while others are entirely chlorotic. The fruit are smaller than the fruit on unaffected trees and abortive seeds are occasionally detected. In the current study, leaf symptoms on Marsh grapefruit dieback-affected trees in orchards of the Murrumbidgee irrigation area of Australia (Figure 26) were in fact so similar to the greening symptoms, that it is quite possible - based on the symptoms alone - to suggest that the two diseases could be considered related if not identical.

The intensity and severity of greening disease symptoms is influenced by a range of external factors of which temperature may be the most significant (Garnier and Bové, 1983; Labuschagne and Kotzé, 1988). Based on analyses of the effects of temperature on symptom expression in affected trees, there are distinct African and Asian forms of the disease (Aubert et al., 1984; Garnier et al., 1987). More severe symptoms are observed at 25°C in plants affected with African greening and at 32°C in plants affected with Asian greening. Varying climatic conditions also affect the distribution of the psylla that transmit the causal agent and so indirectly influence the extent of transmission and infection (Aubert et al., 1984). The symptoms in ACD affected trees are more severe in the cooler months (autumn and early spring) with a reduction in symptoms during the summer months (Broadbent,

1977[1]). In this study, the leaf symptoms were quite marked on many of the Marsh grapefruit trees sampled in September 1990 (early spring). These symptoms were, however, not as evident two months later, in late November. This observation together with the observations by Broadbent (1977[1]), suggest that trees affected with ACD exhibit more severe symptoms at lower temperatures. Hence, ACD may be related to the "African form" of greening.

However, definite diagnosis cannot be based on symptoms alone as similar pathological conditions are often caused by quite different agents (Hayward, 1983). Nematode infections, for example, cause similar disease symptoms (Broadbent, 1977[1]). Greening/dieback has also been likened to a zinc deficiency. Symptom severity and intensity can be affected by co-infection with citrus tristeza virus (Chen et al., 1972). Not all the described leaf symptoms occur simultaneously; the intensity and severity in the discolourations vary with the influences described above and with the progression of the disease within the plant. Some trees may have greening infected branches and apparent nutrient deficient branches on the same tree, both of which appear the same (Fraser et al., 1966). These considerations must be taken into account when sampling field material.

The in vivo characterization of a plant pathogen is, however, limited. Information regarding morphology (cell shape, size and ultrastructure), mode of division, sites of infection and vector relationships can be accumulated and compared with other plant pathogens (Moll and Martin, 1974). The putative greening-associated bacterium in situ, both in affected plant tissues (Laflèche and Bové, 1970; Chen et al., 1971; Broadbent, 1977[1]) and whole infected psyllid preparations (Moll and Martin, 1973; Chen et al., 1973), consists of predominantly long thin filamentous structures, 100 - 300nm wide and up to 3µm long (Laflèche and Bové, 1970; Moll and Martin, 1974). Larger round structures also occur (Chen et al., 1971; Garnier and Bové, 1983) which appeared to be connected to the long thin rods

(Garnier and Bové, 1983). These "round forms" may be a morphological variation of the rod. Similar structures were also reported to occur in the phloem cells of ACD affected plants (Broadbent, 1977[1]). Further, the organism, with the structure as described above, has a characteristic cell wall, approximately 25nm wide, that ultrastructurally resembles that of a Gram-negative bacterium (Moll and Martin, 1974; Garnier and Bové, 1977). Indirect methods (Bové et al., 1980) and cytochemical treatments (Garnier et al., 1984) both confirmed this organism to be a Gram-negative bacterium.

With the development of MIG medium, Sibara (1982) was able to isolate a bacterium from greening affected citrus, which was morphologically similar to the bacterium described in situ. Subsequently, further isolation attempts from greening affected citrus (Duncan, 1985; Garnett, 1985; Mochaba, 1988) have resulted in the accumulation of several isolates that morphologically resemble the long thin rod described in situ. These isolations were made from several greening affected areas in South Africa and from plants infected with South African and Reunion greening and Taiwan likubin maintained in planta in Beltsville, U.S.A. (Mochaba, 1988; Lee et al., 1989).

Several different isolation procedures were investigated by Mochaba (1988) and the chopping method described in sections 2.7.1 and 2.7.2 of this study was concluded to be the most successful. From the work carried out by Mochaba (1988) it is nevertheless evident that the number of isolations of organisms similar in structure to the putative agent, are extremely low. An isolation rate of only about 4% - 10% was obtained. This value represents the number of putative greening-associated isolates obtained relative to the number of isolation attempts made. The isolates were defined by their ability to pass through a 0.45µm filter and possession of the typical cell wall characteristic previously discussed.

The isolates obtained by Mochaba (1988) were further distinguished by the optimal temperature at which they were isolated and subsequently grown, and on their biochemical and metabolic characteristics in culture.

The bacterial isolates NC I, NC III and GL I (identified in the current study as SA03, SA05 and SA01, respectively), as well as NC II, GC I and GRL I (not available in the current study), all of which were isolated from South African greening affected citrus by Mochaba (1988) were isolated at 25°C and grew optimally at this temperature. Others, TZL I, LLL I, LL I (identified in the current study as SA09, SA10 and SA11, respectively) and LC I (not available in the current research) were isolated at 35°C and grew optimally at the higher temperature. Consequently, the bacteria isolated from greening affected citrus in South Africa appear to fall into two groups, categorized by Mochaba (1988) as "low temperature isolates" and "high temperature isolates", respectively. Further isolates obtained from the *in planta* source at Beltsville, U.S.A. (Lee *et al.*, 1989; Mochaba, 1988) included E, I, M, O (identified in the current study as SA06, RE01, TA01 and TA02, respectively), and K (not available in the current study). The E, I and K isolates grew optimally at 25°C and were accordingly grouped with the low temperature isolates by Mochaba (1988), while M and O grew optimally at 30°C (Mochaba, 1988).

Using Analytical Profile Index (API) systems and conventional biochemical tests, Mochaba (1988) grouped the NC I, NC II, NC III, GL I, GC I, GRL I, E, I and K isolates together. Both M and O, although having a higher optimal growth temperature, were grouped with these isolates as their biochemical and metabolic characteristic were the same. TZL, LLL I, LL I and LC I were placed into a separate group sharing similar metabolic and biochemical characteristics.

In the current study, attempts were made to isolate and culture a long thin Gram-negative rod similar to those observed in greening affected citrus from ACD affected leaves and fruit, using the chopping method developed by Mochaba (1988). The number of isolates cultured from Australian dieback which were predominantly Gram-negative rods, less than 0.45µm wide, were few. Of the 87 attempts, 3 isolates were obtained in pure culture, an isolation rate of only 3.5%. Although appearing to be of the correct size and shape under the light microscope, the cell wall ultrastructure of these isolates was not determined conclusively. The isolates were, however, compared to the isolates from greening-affected citrus based on other parameters.

The low isolation rates obtained by Mochaba (1988), and in the current study are not surprising given the nature of the organism and the material used. The putative greening-associated bacterium occurs in low titres in the phloem cells of an affected plant and is erratically distributed (Manicom, 1984). In phloem tissue of ACD only a few organisms are present per sieve tube and only a single infected sieve tube per section was observed (Broadbent, 1977[1]). Higher titres were obtained when the bacterium associated with greening was transmitted to periwinkle (Garnier and Bové, 1983) but these researchers failed to isolate the pathogen in pure culture (Garnier et al., 1987). No attempts have been made to transmit the putative ACD-associated bacterium to periwinkle.

Not only does the isolation procedure have to overcome the problem of releasing the bacteria from the phloem cells, but it also has to minimize any interference by the naturally occurring autotrophic and epiphytic bacteria that prevail on the leaf and fruit surfaces and in plant tissue including citrus. Consequently, stringent control has to be maintained over the surface sterilization procedure of the tissue and the subsequent isolation conditions. Care must be taken to prevent excessive sterilization of the leaf and fruit samples. No growth in the isolation flasks is as

undesirable as growth by a bacterium other than the bacterium investigated. Bacteria, not fitting the description of the putative greening-associated bacterium are nevertheless, still found to occur in the isolation media. Some isolation attempts by Duncan (1985) contained Gram-positive rods. Garnett (1985) reports the occurrence of coryneforms and atypical Gram-negative rods. Mochaba (1988) also observed Gram-positive rods. These "other" bacteria include very large Gram-negative rods, small Gram-positive bacilli and Gram-positive cocci; yeasts were observed in some of the ACD isolation flasks. Are all these other bacteria obtained during the isolation procedure indeed contaminants, or is it perhaps possible that some of these "other" bacteria are actually endophytic on citrus or indeed associated with the greening/dieback disease?

At present this is difficult to answer. The in situ observations of both affected citrus tissues and infected psylla, as previously mentioned, described the long thin rod morphology as characteristic of the putative greening-associated organism. Consequently, isolation attempts from greening-affected citrus by Sibara (1982), Duncan (1985) and Mochaba (1988), were aimed at obtaining in culture, a bacterium fitting this description and having the characteristic cell wall morphology described by Moll and Martin (1974). It is, however, quite possible that other bacteria and indeed viruses may be present in greening affected tissues contributing to the disease but which are overlooked in the search for the characteristic long thin rod. Such bacteria, when isolated, are categorized as contaminants and perhaps wrongly discarded because they do not conform to the described morphology of the greening organism.

As already mentioned, the presumptive causal agent of greening is currently considered by some, but may not necessarily be, a long, relatively thin, bacterium measuring 100 - 300nm wide (Laflèche and Bové, 1970; Moll and Martin, 1974; Garnier and Bové, 1983). Hence, a simple and rapid test was developed by which

the approximate size of the bacteria isolated from greened citrus could be determined (Section 2.7.3). By virtue of its cell width, the bacterium associated with greening is able to pass through a 0.45µm filter which excludes most other bacteria. Bacterial width is a more reliable characteristic than cell length (Trüper and Krämer, 1981). "Ordinary" bacteria such as Escherichia coli generally fall into a 0.4 - 1.0µm x 0.7 - 3.0µm size range (Starr and Schmidt, 1981) and do not pass through a 0.45µm filter. Some bacteria are much larger and some smaller bacteria also occur which measure 0.1 - 0.4µm wide and 0.15 - 1.2µm long (Starr and Schmidt, 1981). As other smaller bacteria do exist, this selection procedure should not be used as a decisive factor in any isolation attempt but rather as an indication that the organism isolated may be of the correct size.

The putative greening-associated bacterial isolates obtained from South African greening-affected citrus and from plants infected with Reunion greening and Taiwan likubin all passed through a 0.45µm filter (Mochaba, 1988). Similarly, in the current study, the isolates from ACD affected citrus were tested for their ability to penetrate a 0.45µm filter. Two of the nine isolates did not pass through the filter. As one of the aims of the current study was to isolate organisms from ACD related to those isolated from greening affected citrus, an additional four isolates were eliminated as they did not react with the antisera raised to the putative greening-associated bacterial isolates.

Criteria such as cell shape, size and staining properties, are all important for the classification of bacteria (Hayward, 1983). In the current study, light microscopic observations were made of the South African, Reunion and Taiwan isolates (Mochaba, 1988), isolates from in planta cultures in quarantine facilities in the US (H.M. Garnett, personal communication) and ACD isolates (Section 3.4.2 of this study) all of which were obtained from plants believed to be affected with greening/dieback. The available isolates were Gram-negative (Figures 2 - 6 and

27 - 29). Based on the light microscope observations, the available isolates appeared to fall into two morphological groups. The cells of South African isolates SA01, SA03, SA05, and SA06; Reunion isolate RE01; Taiwan isolates TA01 and TA02; Australian isolates AU01 and AU02; and American isolate US04, were all small irregular rods that varied slightly in length. With the exception of isolate AU01, all the isolates were Gram-negative. The AU01 isolate, although comparable in morphology to the other isolates was, however, Gram-variable, a phenomenon that occurs with Bacillus and Corynebacteria (Hayward, 1983).

South African isolates SA07, SA09 and SA10; and American isolates US02, US03, US05, US06, US07 and US08, were not morphologically pleomorphic as described above but were generally rod shaped. Filament formation was sometimes seen in these isolates and readily observed for isolate SA07 which became highly filamentous when grown at 35°C. The development of what appear to be sub-terminal endospore-like structures in the SA09, US02, US05, US07 and US08 isolates is interesting.

Sporulation is uncommon in Gram-negative bacteria. The trigger for sporulation is starvation; as essential nutrients become depleted, spore-forming bacteria "undergo progressive morphological, biochemical and physiological changes which culminate in the formation of an endospore" (Szulmajster, 1979). Endospore formation is usually associated with, and used as an identifiable characteristic in Bacillus and Clostridium genera, both Gram-positive organisms (Trüper and Krämer, 1981). Development of these spore-like structures was most notable in isolate SA07 and also readily occurs in the ACD-associated isolate AU03.

The bulging effect in some of the cells illustrated in figure 7, is not quite understood. It occurs sporadically, is not dependant on the stage of growth and occurs in all the available isolates in the current study at one time or another.

Perhaps the phenomenon is related to a breakdown in cell wall integrity that occurs as a result of some form of stress condition. Bulging as a result of a temperature increase in temperature sensitive rod$^-$ mutants of B. subtilis may suggest an explanation. Incorporation of newly synthesized wall constituents from a limited growth zone associated with a modification in the normal cylindrical extension of the cell, results in an increase in the cell diameter and the formation of a bulge in B. subtilis (Burdett, 1979). From in situ observations, the peptidoglycan layer of the bacteria seen in the phloem cells of greening affected plants was thought to be very thin (Garnier et al., 1984). Hence it is possible that, if the isolated organisms currently studied are indeed the causal agents of greening/dieback, then any stress caused in culture could disrupt the thin peptidoglycan layer and affect the overall integrity and stability of the cell wall.

The putative greening-associated bacterium is a long thin rod in situ (Laflèche and Bové, 1970; Chen et al., 1971; Moll and Martin, 1974; Garnier and Bové, 1983). It is evident from the light microscope observations, however, that the organisms isolated in culture, although fitting the thin rod description, do vary in length. Cells of the SA01, SA03, SA05, TA01 and TA02 isolates, described as small thin irregular rods based on light microscope observations, become more pleomorphic with growth developing terminal bulges and appearing club and dumb-bell-shaped (Figure 21). These irregularities in cell shape were further accentuated with growth at a higher temperature (Figure 22). While the SA09 and SA10 isolates maintained the rod shape morphology throughout the growth cycle (Figure 23), all of the SA01, SA03, SA05, TA01, TA02, SA09 and SA10 isolates followed through a growth cycle that ended with the development of round forms as the cultures aged and the nutrients in the medium became depleted. Furthermore, this progressive development differed with the temperature at which the isolates were grown. The apparent progressive development of the round form from the long thin rod as nutrients become depleted (Figures 21 and 24), may suggest that the observed

structures indeed are related to the greening organism as defined by the in situ observations in which the round form structures are considered "different aspects of the same organism" (Garnier and Bové, 1983).

The effects on growth in a medium as complex as MIG would be quite varied as each of the constituents becomes depleted and the cell wall integrity of the bacterial cell is affected. Cell shape is maintained by the peptidoglycan layer between the cytoplasmic and outer membranes. Observations of E. coli cell wall during the growth cycle suggest that although the murein becomes more crosslinked during the stationary phase, the glycan chains become shorter (Pisabarro et al., 1985). The strength of this network is unknown but during rod to sphere changes in a Vibrio sp., the spheres develop from the degradation, but not complete removal, of the peptidoglycan layer (Baker and Park, 1975). Plasmolytic effects have been suggested as a cause for round form development based on in situ observations of the bacterium in the phloem of greening-affected plants (Garnier and Bové, 1983). It is likely that the round forms observed in this study develop as a result of a breakdown in the integrity of the cell wall of the long thin rod.

As previously mentioned, the effects of temperature on symptom expression in greening affected citrus resulted in the distinction of an "African form" and an "Asian form" of the disease. Mochaba (1988) observed that some of the bacterial isolates from South African greening affected citrus and used in this study, were isolated at 25°C and grew optimally at this temperature. Others were isolated at 35°C and grew optimally at the higher temperature. Consequently, the bacteria isolated were re-categorized into "low temperature isolates" and "high temperature isolates" respectively. It is surprising therefore, that the shape of the growth curves for SA01, SA03 and SA05 isolates (low temperature isolates as defined by Mochaba, 1988), the SA09 and SA10 isolates (high temperature isolates as defined by Mochaba, 1988), and TA01 and TA02 isolates (isolates which grew optimally at

30°C) were the same in the current study (Figures 14 - 20). This observation may be attributed to the use of a different approach taken by Mochaba (1988) to record cell growth. Mochaba (1988) determined the optimal temperature of growth by inoculating plates with different concentrations of a log phase culture. These plates were then incubated for 5 days at different temperatures and the number of colonies scored. The temperature at which the plates supported a larger number of colonies was recorded as the optimal temperature of growth.

In the current study, the growth characteristics of several isolates were investigated in liquid cultures at both 25°C and 35°C. These two temperatures were selected as they represent the temperatures at which symptoms were more severe for African and Asian greening, respectively (Garnier and Bové, 1983; Labuschangne and Kotzé, 1988; Mochaba, 1988). Although Asian greening symptoms occur at 30°C - 32°C, the growth experiments in the current study were conducted at 35°C to allow for a more clearcut margin between the two temperatures. Also 35°C represents the optimal temperature of growth for the high temperature isolates defined by Mochaba (1988).

With the exception of isolate SA09, the SA01, SA03, SA05, SA10, TA01 and TA02 isolates had similar growth rates during the exponential phase at both 25°C and 35°C (Figures 14 - 20). However, isolates SA01, SA03, SA05, SA09, SA10, TA01 and TA02 attained higher bacterial titres at 25°C than at 35°C. If these organisms are indeed the causal agents of the greening disease, their greater replication at 25°C could be associated with the increased severity of the disease symptoms of African greening.

An analysis of variance (ANOVA) was performed on both the exponential and stationary phase data of the growth curves (Appendix VIII). Conclusions regarding

the similarity in the exponential rate of growth at 25ºC and 35ºC and the higher bacterial titres attained at the lower temperature were supported.

The effect of age and nutrient availability on the morphological variation of the putative greening-associated bacterium was not only observed in liquid cultures but was also evident in colony cross sections. Electron microscopy of whole colony cross-sections of isolates SA01, SA03 and SA09 complemented observations made by Ariovich and Garnett (1985). Young colonies consisted predominantly of long thin rods evenly distributed throughout the colony (Figure 11). As the colony matured and the nutrient availability towards the centre of the colony became depleted, both the cell morphology and distribution changed from the surface to the centre of the colony (Figure 12). Comparable observations of a rod to sphere morphological variability within a colony, considered to be induced by a nutrient deficient condition, have been made by Ng et al. (1985). Unlike the TEM observations by Ariovich and Garnett (1985), no spores were evident in the plate cultures. This may be attributed to the fact that the colonies were grown at 25ºC and not at 35ºC at which temperature sporulation occurs in some of the isolates (Mochaba, 1988). However, other studies have indicated that spores do occur in isolate SA09, while isolates SA01 and SA03 have never been observed to sporulate (H.M. Garnett, personal communication).

When the isolates derived from greening/dieback affected citrus were grown at 25ºC on solid MIG medium, two colony types were observed. The first, characteristic of isolates SA01, SA03, SA05, SA06, SA11, RE01, TA01, TA02 and US04, was a circular, convex colony with an entire margin, 1 - 4mm in diameter and with a variable yellow pigmentation (Figures 2 - 4). The second colony type, characteristic of isolates SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 was similar to the first with the exception that it was flatter and had a creamy brown colouration (Figures 5 and 6). Mochaba (1988) described both the above

colony types for the Asian isolates M and O (identified in the current study as TA01 and TA02, respectively). At 25°C colonies of these two isolates were yellow while at 35°C the creamy/brown, or a creamy-tan-khaki colony type, as described by Mochaba (1988), occurred.

Several isolates stored at 4°C, produced very bright yellow or orange, almost fluorescent colonies. These colonies reverted to the more characteristic creamy yellow colouration with subculture and incubation at 25°C. There was no difference in the bacterial cell morphology in stained smears of the differently coloured colonies.

Plant pathogenic bacteria produce a variety of pigments many of which can be used diagnostically. They are useful characteristics for identification of some Xanthomonas, Corynebacteria and Erwinias (Hayward, 1983). Unfortunately, the variability in colony colouration with age and temperature complicates the use of colony pigments to identify the putative greening-associated bacteria, although this characteristic distinguishes the isolates into two groups. Occurrence of a creamy/white colony among the yellow colonies in plate culture of isolate SA03 (Figure 8) further reflects problems associated with classification based on colony morphology alone. The creamy/white colony was maintained in pure culture (Figure 9), did not revert to the yellow colony type (Figure 10) and differed only with respect to its pigmentation. The ultrastructural, molecular and serological characteristics of the bacteria of the pale/white colony were identical to the bacteria from its yellow counterparts.

From the morphological observations it appears that there are two distinct groups to which the bacteria isolated from greening and dieback affected samples can be allocated. This distinction is, however, not clear cut, as some degree of variability in cell shape or colony pigmentation does occur within the groups. Based on the

colony characteristics and the structural characteristics determined during the growth cycles by light and electron microscope observations, isolates SA01, SA03, SA05, SA06, SA11, RE01, TA01, TA02, and US04 may be grouped together and will subsequently be referred to as the Group 1 isolates, and isolates SA07, SA09, SA10, US02, US03, US05, US06, US07 and US08 may be grouped together and will further be referred to as the Group 2 isolates.

It is interesting to note at this point that, with the exception of the bacteria isolated from in planta greening cultures in the greenhouse in the US, included in the current study, the two colony type groups coincide with the previously discussed temperature related and metabolic groupings initially allocated to these cultures by Mochaba (1988).

Furthermore, the previously discussed distinction between the biochemical and metabolic properties of NC I, NC III, GL I, TZL I, LLL I, E, I, O, M (identified in this research as SA03, SA05, SA01, SA09, SA10, SA06, RE01, TA01 and TA02, respectively), NC II, GRL I, GC I and LL I (which were not available in the current research) from LLL I, TZL I, LL I and LC I (LLL I and TZL I were identified in the current study as SA10 and SA09, respectively, LL I and LC I were not available) by Mochaba (1988) was also observed in Biolog metabolic assay performed in the current study (Section 2.4).

The ability of the isolates from greening /dieback affected citrus to metabolize various carbon sources was investigated (Section 2.4). After 24 hours, no growth was observed for the Group 2 isolates. The lack of growth by the US isolates may be attributed to the fact that these isolates were tested immediately after isolation from plants while the other isolates were subcultured 5 - 6 times. However, this reasoning does not apply to US04, suggesting that the other US cultures may indeed not have been unable to utilize the various carbon sources provided. Growth

occurred for isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02, AU01, AU02 and US04 (Section 3.3) and with the exception of AU02, similar metabolic patterns obtained. Hence, the Group 1 isolates and the AU01 and US04 isolates could possibly be considered closely related organisms.

The observed metabolic similarity of the Group 1 isolates with Clavibacter michiganense subsp. michiganense and Clavibacter michiganense subsp. insidiosum is interesting (Section 3.3). The genus Clavibacter constitutes the plant pathogenic species of coryneform bacteria (Collins and Bradbury, 1986). These are all Gram-positive, aerobic, non sporeforming pleomorphic rods approximately 0.4 - 0.75µm wide and 0.8 - 2.5µm long (Vidaver, 1982; Collins and Bradbury, 1986) and hence morphologically very different from the Group 1 isolates all of which are, with the exception of AU01, which is Gram-variable, Gram-negative rods less than 0.3µm wide. It is also interesting that a bacterium isolated from greening affected citrus in South Africa by Chippindall and Whitlock (1989[2]) was also identified as a Clavibacter sp. suggesting that a Clavibacter sp. may indeed be associated with greening affected citrus.

In the same way that metabolic tests allow an organism to be "fingerprinted" according to its ability to use certain carbon sources (Bochner, 1989), so too can an organism be identified according to its cellular proteins separated by SDS-PAGE.

Closely related organisms may be assumed to have similar or identical kinds of cellular proteins (Jones and Krieg, 1986). Cellular proteins separated by SDS-PAGE result in a characteristic protein pattern or "fingerprint" that reflects the genetic background of a bacterial strain which can then be compared with similar fingerprints of other bacterial strains as a measure of genetic relatedness (Jones and Krieg, 1986). Hence, the available bacterial isolates obtained from both greening and dieback affected citrus were further investigated by SDS-PAGE.

In a preliminary study, Mochaba (1988) compared the protein patterns of South African isolates NC I and LLL I (identified in this study as SA03 and SA10, respectively), GC I (an isolate comparable to SA01) and LC I (not available in this study) and a Taiwan isolate M (identified as TA01). The cell wall/outer membrane protein patterns of these isolates were similar with major proteins in the apparent molecular weight ranges of 96K - 94K, 79K and 69K - 66K shared by all the isolates (Mochaba, 1988).

In the current study, whole cell protein patterns were established for the Group 1 isolates consisting of the South African isolates SA01, SA03 (both the yellow and white variants), SA05 and SA06, Reunion putative greening-associated isolate RE01, Taiwan putative likubin-associated isolates TA01 and TA02 and US04 isolate. Patterns for the bacteria isolated from ACD affected citrus morphologically resembling the putative greening-associated bacterium, that is, isolates AU01, AU02 and AU03, were also included.

The similarity of the patterns obtained for isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 (Figure 33) tends to suggest that the Group 1 isolates are the same species, complementing the previous observation regarding the similarity of the metabolic characteristics of these isolates. The additional protein in isolates SA03 and TA02 with an apparent molecular weight of 38K - 39K could suggest that isolates SA03 and TA01 are possibly subspecies of the Group 1 isolates. It furthermore appears from the protein patterns of isolates AU01 and US04, that these bacteria could be related to isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02. The whole cell protein pattern of isolate AU02 although appearing similar to those of isolates AU01 and US04 in the 43K - 67K range, does not resemble that of the isolates in the higher and lower molecular weight ranges. The AU02 whole cell protein pattern was, however, very similar to the patterns of both C. insidiosum, and C. michiganense suggesting that it is related to these organisms

and that all three differ from the Group 1 isolates. Different patterns were observed for isolates SA09/SA10 and SA07/AU03, all four belonging to Group 2. Hence it is possible that there are two subgroups within Group 2. This will, however, have to be investigated further as protein patterns were not established for all the Group 2 isolates. The pigment variation of SA03 cultures did not affect the protein pattern, the yellow and white colony variant having the same pattern.

The protein patterns for A. radiobacter, E. coli, P. aeruginosa and S. typhimurium did not share any similarities with either the Group 1 or Group 2 isolates. Only a single protein of approximately 33K was observed for B.subtilis, suggesting that although the preparation for SDS-PAGE described in section 2.8.1 results in satisfactory protein patterns for the Gram-negative bacteria, it will have to be modified for Gram-positive cells. Similarly, twice the amount of protein had to be loaded onto gels for C. insidiosum, and C. michiganense so that clear patterns could be resolved using the staining techniques employed in the current study. It appears, however, that one of the isolates from ACD affected material, namely AU02, may be a Clavibacter sp.

The protein patterns discussed for the Group 1 isolates above were comparable to the patterns investigated by Mochaba (1988), although the patterns obtained in the current study contained more bands than the patterns obtained by Mochaba (1988). This increase in band number for similar isolates may be attributed to either altered preparative procedures and/or to more passaged isolates.

In addition to the protein bands described by Mochaba (1988), major protein bands in the apparent 38K - 40K, 29K - 32K and 8K - 10K molecular weight ranges were also shared by the isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02 and US04.

The age of the culture did not affect the protein pattern of either isolate SA01 (a South African putative greening-associated isolate) or isolate TA02 (a Taiwan putative likubin-associated isolate) suggesting that the overall protein constitution of the long thin rod and round form of these two isolates remains similar (Figure 49). The band numbers and positions remained constant throughout the different stages of growth, although the relative amounts of protein in the bands appeared to increase when older cultures were analysed.

During culture preparation for SDS-PAGE, aliquots were taken for protein estimation so that comparable amounts of protein could be loaded onto the gels. These aliquots were taken after the cell suspension had been sonicated and before the cells were centrifuged, the pellet resuspended in PBS and further diluted 1 : 3 in sample buffer (Section 2.8.1). As soluble proteins were discarded with the supernatant, the amount of protein actually loaded onto the gels was far less than that originally estimated. The protein patterns of the isolates could nevertheless be investigated as the relative amounts loaded still allowed comparable protein patterns to be obtained for the isolates. However, as will be observed later, the detection of protein bands in western blots of plant preparations spiked with bacterial cells was greatly reduced by the lower protein amounts actually loaded onto the gels. It was also noted during the current study that the pellets of older cultures were more soluble in the sample buffer and consequently more intense bands were observed at the later stages of growth as was evident for isolates SA01 and TA02 (Figure 49).

The fact that the protein patterns were conserved during growth, suggested that it could be feasible to develop a detection system that was based on the antigenic surface properties of the bacteria. Antisera raised against the long thin rod of the exponential phase of growth should be able to detect the several morphological forms

observed in culture and in situ, if these isolates are indeed related to the causal agents of greening and dieback.

Serological techniques have been used extensively for the detection and identification of plant pathogenic bacteria (Schaad, 1979) and play a valuable role in bacterial taxonomy. Two criteria of value in serological characterization include the comparative observations of bacterial cell surface antigenic determinants and the detection of homologous proteins in different bacteria with antisera raised to a purified protein (Jones and Krieg, 1986). Methods such as ELISA's are "simple, rapid and sensitive assays for the immunological characterization of phytopathogenic bacteria obtained from either cultures or diseased tissues" (Lazarovitz et al., 1987). Most of the commonly isolated bacteria are classified to the genus level by symptomological observations, Gram stain characteristics, colony morphologies and biochemical tests. These characteristics may be variable and often unreliable as separate entities. Serological characterization, however, provides a final, more definite confirmation in the identification and classification to the species level (Schaad, 1979).

Polyclonal antisera were successfully raised against three South African isolates (SA01, SA03 and SA07), the former two being categorized as "low temperature" isolates by Mochaba (1988) and Group 1 isolates in the current research, and the latter as a "high temperature" or Group 2 isolate, and a Reunion isolate (RE01) also a Group 1 isolate in the current study. The purified IgG of these antisera were subsequently used in slot-blot immunoassays. Not only was there reaction of the homologous antigen but there were several cross reactions with the other isolates obtained from greening and dieback affected citrus.

Cross reactions observed with polyclonal antisera are often a limiting factor. Indeed the specificity of an antiserum depends on the combined specificities of the

constituent antibody molecules (Catty, 1988). When complex antigens such as whole bacterial cells are used to stimulate an immune response, a multiplicity of antibodies may be present in the antiserum each of which may differ in their relative concentration and affinity for antigen-antibody bindings. More commonly, however, antigenic determinants are shared between molecules. Antisera raised to one antigen may, therefore, cross react with another. The antiserum must then be rendered specific to the inducing antigen by absorption with the antigen containing the shared antigenic determinants.

The specificity of reaction in serological assays is dependent on the methods employed in raising the antisera and the procedures subsequently applied in the reaction systems. The conditions under which the bacteria are grown influence the composition of the cell wall and extracellular products (Poxton and Blackwell, 1986). Antiserum specificity depends on the antigenic preparation used as an immunogen and on the animals immunized (De Boer, 1982). The specificities of the reactions in the assays are dependent on the incubation times and the antibody and antigen concentrations (Lazarovitz et al., 1987). Indeed the conditions under which serological assays are carried out are so variable that standardizations of the procedures are required (Schaad, 1979). A typical example was observed in the current study. Increasing the amount of isolate RE01 whole cell protein loaded for SDS-PAGE and subsequent reaction with anti-SA01, anti-SA03 and anti-RE01 IgG in western blots, resulted in serological reaction patterns that contained far more bands (Figure 40) than when smaller protein quantites were used (Figures 34 - 36).

In the procedure for antigen preparation described by Duncan (1985), an unencapsulated bacterium was a more efficient immunogen than a capsulated bacterium. Antisera raised against capsulated bacteria were less specific and this decreased specificity attributed to the foetal calf serum in the medium. This

problem appears to be common and also complicates the preparation of specific antisera to mycoplasmas (Senterfit, 1983). Serum proteins in the growth medium adsorb to the organisms resulting in the production of antibodies to the serum proteins during immunization rather than to the bacterial determinants (Senterfit, 1983). Foetal calf serum is a requirement for growth on primary isolation of bacteria from greening affected citrus (Sibara, 1982). Once the cultures have become established, however, the bacteria grow satisfactorily in MIG from which the foetal calf serum component was omitted (Mochaba, 1988). The presence of foetal calf serum in complete MIG medium not only interferes with light microscope observations by producing globules during fixing, which hinder morphological observations, but also affects the Gram staining properties of the bacteria. It further interferes with serological assays by cross reacting non specifically with the antisera. Consequently the procedure for antigen preparation used in the current study was a modification of that described by Duncan (1985). The bacteria for immunization were grown in incomplete MIG medium. In addition, all the subsequent serological assays were performed with cultures grown in medium from which the foetal calf serum was omitted.

Because of the heterogeneity in specificity of polyclonal antisera, the pre-immune sera of the animals to be immunized in this study had to be tested for non specific cross reactions. The suitability of these antisera in the immunoassay for which they were intended was also investigated.

The detection of isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02, and US04 (all Group 1 isolates as defined earlier) in slot-blot imunoassays was observed to be equally specific when the bacterial suspensions spotted were adjusted by absorbance and reacted with the anti-SA01, anti-SA03 and anti-RE01 IgG (Section 3.6). Weaker reactions were observed with isolates US07, US08, C. insidiosum and C. michiganense (Figure 31). Reactions did not occur with the corresponding pre-

immune IgG's at the same concentration and reacted with the Group 1 isolates prepared as described above. However, reactions did occur with isolates US07, US08, C. insidiosum and C. michiganense. Standardizing the bacterial suspensions by absorbance was preferred. When the suspensions spotted were adjusted according to cell numbers, more cross reactions were observed with the laboratory cultures and the pre-immune sera (Figure 30). These pre-immune cross reactions were, however, greatly reduced when the bacterial suspensions were standardized by absorbance. The reactions of the Group 1 isolates were more specific using this method of preparation and all the isolates reacted with the anti-SA01, anti-SA03 and anti-RE01 IgG's. Anti-SA07 reacted with the homologous antigen and with the Australian isolate AU03 equally well and reacted weakly with isolates SA09, SA10, US07, US08 and C. michiganense (Figure 32).

Serological cross reactions are common in Gram-positive bacteria and observed among Corynebacterium species (De Boer, 1982). Similarly, common antigenic determinants would be expected to occur in Gram-negative bacteria. Corynebacterium sepedonicum not only cross reacts with other Gram-positive bacteria but also with some Gram-negative bacteria (De Boer, 1982). The reaction of Clavibater michiganense subsp. michiganense with the anti-SA01, anti-SA03, anti-RE01 and anti-SA07 IgG's and Clavibacter michiganense subsp. insidiosum with the anti-SA01, anti-SA03 and anti-RE01 IgG's suggests that there is either a serological relationship between the putative greening-associated bacteria and this bacterium or that there are certain shared antigenic determinants detected non specifically. The latter is supported by the observed reactions with the pre-immune IgG. A relatedness between the Clavibacter sp. and the Group 1 isolates is based on the metabolic observations previously discussed but not supported by the protein pattern observations. Further evidence obtained in western blots suggest that the above observed cross reactions might indeed be non specific. The E. coli and B. subtilis cross reactions may be attributed to previous exposure of the immunized

animal to these bacteria, as positive pre-immune IgG reactions were observed with the two isolates.

The relatively strong serological cross reactions of isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02, and US04 with the anti-SA01, anti-SA03 and anti-RE01 IgG's suggest that these isolates belong to a serologically related group, sharing the same surface antigens. Similarly, the strong cross reactions of isolates SA07 and AU03 with the anti-SA07 serum suggest that these two isolates are related. The weaker cross reactions observed for isolates SA09, SA10, US07 and US08 with the anti-SA01, anti-SA03 and anti-RE01 sera may be attributed to some shared surface antigens. Although cross reactions occurred for the Australian isolates AU01, AU02 and AU03 with the anti-SA01, anti-SA03 and anti-RE01 IgG's in the first slot-blot immunoassay (Section 3.6), no reactions occurred when the antigen concentration was lowered and the slot-blot immunoassay repeated. This observation may be attributed either to a non specific binding by the antisera to the more concentrated antigen preparation; or to the fact that only a few antigenic determinants are shared with the other putative greening-associated bacteria, the specificity for which was lost when a more dilute bacterial suspension was used. However, the SDS-PAGE protein patterns of the AU01 and AU02 isolates resemble the patterns of the Group 1 isolates.

The use of the anti-SA01, anti-SA03, anti-SA07 and anti-RE01 IgG's in identifying greening affected plants was also investigated. Mild positive reactions were at first observed with healthy tissue. These reactions may be attributed to either a non specific binding of the antiserum, to the existence of possible shared antigens between the bacteria and the plant material or to the presence of endophytic bacteria that are the same or related. The sharing of antigens between pathogens and hosts is common (De Vay and Adler, 1976). The binding of plant tissue whether specific or non-specific was removed when the antisera were cross absorbed with healthy

tissue. Cross absorbing with the same citrus variety is preferred (R.F. Lee, personal communication; H.M. Garnett, personal communication). Hence, all the IgG used in the slot-blot immunoassays were cross absorbed with preparations from healthy citrus and tristeza-infected citrus to reduce some non-specific binding.

Slot-blot immunoassay screening for the identification of greening by H.M. Garnett (Personal communication) with the anti-SA01, anti-SA03 and anti-RE01 IgG raised in this study, cross absorbed with a preparation from healthy citrus and tristeza-affected citrus reacted with preparations from citrus affected with African and Asian greening maintained in planta in Beltsville, U.S.A. Anti-SA07 reacted with preparations from some in planta sources but the diagnostic values of these reactions were limited. Healthy control leaf preparations yielded negative results with the cross absorbed sera and corresponding cross absorbed pre-immune IgG for the four sera in all tests.

The identification of Australian dieback affected citrus was consequently investigated in this study using antisera raised to the African and Reunion cultures and cross absorbed with preparations from healthy grapefruit tissue. Based on the positive results (Figure 51), there may be a serological relatedness between the causal agents of the two diseases. Radiolabelled second antibodies were favoured in the procedure as the plant pigment often hindered the interpretation of the colour development of the alkaline phosphatase system. The anti-SA01, anti-SA03 and anti-RE01 IgG did not react with the non symptomatic samples taken from neighbouring "healthy looking" trees in the orchards from which the ACD samples were collected. There were no reactions with the pre-immune IgG of the antisera cross absorbed with healthy grapefruit tissue preparations and reacted with either the affected or "healthy" samples in a slot-blot immunoassay.

Other serodiagnostic systems more sensitive and specific than the slot-blot immunoassays, were also investigated. By exposing the protein patterns obtained by SDS-PAGE, to antisera, specific serologically reactive bands can be visualized and characterized according to molecular weight and composition (Catty, 1988). Indeed, immunostaining not only allows for the testing of antibody specificity, but shared antigenic bands in patterns of the same or different preparations can also be determined. The combined application of electrophoretic separation and serological reaction in western blots have contributed greatly towards the reliability of bacterial species and strain differentiations.

Western blots were consequently investigated in the current study in which the protein patterns obtained for the isolates by SDS-PAGE (Figure 33) were transferred to nitrocellulose and reacted with the purified IgG preparations as described in section 2.9.7 and 2.9.6.

Western blot analyses of the cultured putative greening/dieback-associated isolates serologically relate isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02. Indeed, the similarity of the serological reaction patterns using the anti-SA01, anti-SA03 and anti-RE01 IgG (Figures 34 - 36) suggest that the isolates are identical. Anti-SA01 reacted with major bands with apparent molecular weights of 75K - 79K and 38K - 40K and with two bands with apparent molecular weights of 36K and 23K (Figure 34). Anti-SA03 reacted with major antigenic bands of apparent molecular weight 70K - 78K and bands with apparent molecular weights of 51K, 39K and 35K (Figure 35). Anti-RE01 reacted with bands with apparent molecular weights of 116K, 104K, 90K, 73K, 52K, 39K and 34K (Figure 36). The major antigenic bands of isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 are not only serologically reactive, but also react equally well with the three IgG's tested. None of these major bands were detected with the corresponding pre-immune IgG.

The serological reactions of isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 were very different from those of isolates SA07 and AU03 which were identical when reacted with the anti-SA07 IgG (Figure 37). It appears, therefore, that although geographically delimited, both these isolates are serologically identical, suggesting that the SA07 and AU03 isolates are the same bacterium and supports the previous allocation of these two isolates into a subgroup of the Group 2 isolates. Again further work is needed to clarify the serological relationships of the other Group 2 isolates. Emphasis was placed on Group 1 as this group of isolates caused symptoms in artificially inoculated seedlings and consequently were favoured for the establishment of a serological detection system.

Only a single protein (Section 3.8) in isolate US04 reacted with anti-SA01, anti-SA03 and anti-RE01 IgG's. US04 was isolated from a plant grafted with material from seedlings injected with SA01. A serological reaction/western blot similar to SA01 would therefore have been expected for US04 if this organism was the injected organism. The observed difference in the serological reaction/western blot patterns may possibly be attributed to several factors. Bacterial virulence is lost with subculture in vitro and may be due to changes in the cell wall constitution (Brown and Williams, 1985). "Growth for several generations in a standard bacterial medium produces bacteria with virulence, immunogenic and susceptibility properties greatly different from those obtained in vivo" (Brown and Williams, 1985).

The bacterial glycocalyx is lost with subculture in vitro (Costerton et al., 1981). In Gram-negative bacteria, lipopolysaccharides (LPS) are consequently exposed resulting in antigenic properties which are quite different from that of the glycocalyx enclosed cells (Costerton et al., 1981).

Isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 were subcultured at least 5 - 6 times *in vitro* and consequently a change in the surface properties may be expected. Isolate US04 was available as a first subculture. If this isolate is the same as the SA01 isolate injected, it may be assumed to be more representative of the putative greening/dieback-associated isolates with respect to serological characteristics. Bacterial virulence lost *in vitro* may be restored by passage *in vivo* (Brown and Williams, 1985). When isolate SA01 was injected into plants it had already been subcultured several times. Greening-like symptoms were observed in the injected plants and graft transmission of the agent causing these these symptoms obtained (H.M. Garnett, personal communication). Hence it is possible that the many serologically reactive bands observed in the SA01, SA03, SA05, SA06, RE01, TA01 and TA02 isolates were acquired with *in vitro* subculture and that only the 38K - 40K protein detected in these isolates and in the US04 isolate is a reliable surface determinant for serological detection of the organisms *in situ*.

If this assumption is correct, the reaction of this protein in isolate AU01 tested after 3 passages, suggests that AU01 is serologically related to the putative greening-associated isolates SA01, SA03, SA05, SA06, RE01, TA01, TA02 and US04. Similarly, the reaction of a single antigenic band in isolate US02 with the anti-SA07 IgG (Figure 38) suggests a relatedness between this isolate and the SA07/AU03 subgroup of the Group 2 isolates and may further relate this subgroup to the Group 1 isolates.

The major antigenic bands in western blots of whole cell protein preparations of isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 reacted with anti-SA01, anti-SA03 and anti-RE01 IgG were used to raise a monospecific polyclonal antibody (Section 3.10). Further, although the overall serological reactivity varied with the stage of growth of the SA01 and TA02 isolates (Figure 50), the observed major

antigenic proteins occurred in all the stages of growth suggesting the occurrence of similar serological reactions in both the long thin rods and round forms. The pre-immune sera of the particular animals used to raise antisera to these protein bands, were screened in a western blot for non specific reactivity with the protein bands later to be used as the immunogen.

Of the five antisera raised, only anti-C was specific for the 38K - 40K protein to which it was raised. The corresponding pre-immune serum did not react with this protein band although several proteins in whole cell protein preparations of more commonly occurring Gram-negative bacteria did react (Section 3.10.2). Prior exposure of the rabbit to these or related organisms would have stimulated the immune response to produce antibodies to these bacteria. As all the rabbits were maintained in the same animal house under identical conditions, it is therefore not surprising that the anti-C serum above, the sera raised against SA01, SA03, SA07 and RE01, and the corresponding pre-immune sera previously discussed did have weak reactions with commonly occurring Gram-negative and Gram-positive bacteria such as E. coli and B. subtilis. The reaction of the 38K - 40K protein band was markedly enhanced in western blots (Section 3.10) when reacted with the more specific anti-C IgG at a concentration as low as 0.1µg/ml. Anti-C IgG readily reacted with the 38K - 40K protein band in the Group 1 isolates (Figure 42) obtained from greening and dieback affected citrus; which were suggested to be related in the Biolog assay (Section 3.3); and, which had similar protein patterns in SDS-PAGE (Figure 33).

The 38K - 40K protein band was not detected with the anti-C IgG in western blots of either the laboratory cultures or plant pathogenic bacteria tested including B. subtilis, E. coli, P. aeruginosa, P. fluorescens, S. typhimurium, C. insidiosum and C. michiganense (Figure 43).

The failure to detect any protein bands with the anti-C IgG in the western blot patterns of either C. insidiosum and C. michiganense further confirms the earlier protein pattern observations suggesting that these two organisms are not related to the Group 1 isolates. From SDS-PAGE the amount of protein required to obtain a pattern for either C. insidiosum and C. michiganense, was at least twice that needed for preparations from the putative greening/dieback-associated isolates. Consequently, an argument against the above statement could be made: As only 25µg of protein was reacted with anti-C IgG in a western blot (Section 3.10.2), it may be assumed that the amount of protein loaded was too low to be detected. Hence, the statement regarding the relatedness between the Group 1 isolates and Clavibacter sp. could be incorrect. However, in a previous observation (Section 3.10.1), a 38K - 40K protein band specifically reacted in western blots of 35µg protein samples of all the Group 1 isolates reacted with anti-C IgG at 0.1µg/ml. Although the amount of protein loaded for both the C. insidiosum and C. michiganense cultures was 10µg less, the anti-C IgG was used at a 1.0µg/ml, that is, ten times more concentrated. If the 38K - 40K was present in either C. insidiosum or C. michiganense, it should have been detected. It is possible, however, that other serologically reactive proteins may occur in both the Group 1 isolates and the Clavibacter sp. that could apparently relate these organisms serologically. It should be noted that isolate AU02 which yielded almost identical protein patterns to the Clavibacter sp. (Figure 33), did not react with any of the antisera raised against whole cells of the Group 1 isolates in western blots where 50µg of isolate AU02 preparation was loaded (Figures 34 - 36).

An antiserum developed for use in one assay, does not necessarily perform as efficiently in another and may loose its sensitivity altogether. This occurred with the anti-C IgG. When the anti-C IgG was applied in slot-blot immunoassays, the reactivities of both the anti-C IgG and the corresponding pre-immune IgG were the same (Section 3.10.3, Figure 45). The positive reactions in the slot-blots may be

attributed to shared antigenic determinants present on the whole cells and recognized by both sera. The use of SDS in electrophoresis affects protein charge and structure and consequent changes the antibody-antigen reactivity is expected (Anderton and Thorpe, 1980).

Because of the reactions of both the anti-C IgG and corresponding pre-immune IgG with the isolates, the antiserum could not be used in the identification of greening and dieback affected citrus samples in a slot-blot immunoassays. Consequently, attempts were made in the current study to process plant material for analysis in western blots. There were no apparent reactions with any of the anti-SA01, anti-SA03, anti-SA07, anti-RE01 and anti-C IgG's. This was assumed to be attributed to very low levels of detection.

Different approaches were then investigated in an attempt to determine the bacterial concentration required in plant tissue for positive detections in western blots.

The specific reactions observed in slot-blot immunoassays with the anti-SA01, anti-SA03 and anti-RE01 IgG (Figure 51) suggest that although the putative greening-associted bacteria occur in low titres in the phloem sieve tubes, they could still be detected. Two questions consequently arise: Why are there no antigenic protein bands detected in western blots of preparations from affected plant material that were observed to give positive results in slot-blot immunoassays; and, why is the level of detection in western blots of spiked tissue limited to $>1 \times 10^6$ cells/well when at least 1×10^5 cells/ml (effectively 1×10^3 cells/spot) have been detected using the same sera and plant preparations in slot-blot immunoassays? The answer to both questions may be found in the preparation of the whole bacterial cells for electrophoresis. As previously mentioned, the protein estimation and/or cell concentrations of the bacterial preparations were determined before solubilization. Although sonicating the bacterial suspensions did cause some cellular breakage,

whole cells were still present that did not necessarily solubilize in the sample buffer with boiling but did account for the total protein content or cell concentration of the preparation. The soluble protein in the supernatant was also discarded after centrifugation. Consequently, the amount of protein that was actually loaded onto and separated in the gel system was far less than originally determined and so the amount of protein available for reaction with the antisera was greatly reduced.

The sensitivity of reaction with preparations from affected field samples was further limited to the extent that the plant tissue could be concentrated without the preparation physically interfering with the electrophoretic separation or antibody-antigen reaction in the blots. In the current study, leaf midribs were homogenized 1/5 (w/v) and 1/10 (w/v) in PBS. Both these preparations were further concentrated before sample buffer was added and the preparations boiled in the presence of SDS. In both cases, the resulting preparations were very viscous and took a long time to penetrate the gel during electrophoresis. This may explain the two protein bands of apparent molecular weights 36K and 38K in the western blot of plant tissue preparation spiked with the pooled culture supernates of isolates SA01, SA03, SA05, SA06, RE01, TA01 and TA02 and reacted with the anti-C IgG (Figure 56). In a previous observation anti-C IgG, only detected a single protein band in the medium in which isolate RE01 was grown that was comparable to the protein band detected in the whole cell preparation of the same bacterium (Figure 46). The changes in the physical conditions caused by the slow penetration of the preparation spiked with the culture supernates may have resulted in the separation of two observed protein bands that would normally occupy the same position.

The presence of excessive non-phloem plant tissue was greatly reduced using a modification of the method described by R.F. Lee (Personal communication) for the extraction of blight proteins from xylem. The procedure described in section 2.9.6.4 eliminates the presence of plant tissue other than the phloem constituents

and hence, only phloem exudates are screened for the presence of the greening/dieback-associated bacterium. Based on preliminary results in this study (Section 3.12.5), protein bands in western blots of ACD affected phloem exudate preparations reacted with anti-SA03 and anti-C IgG. These bands were extremely faint and consequently conclusive deductions could not be made. However, application of this method for the identification of greening and dieback affected citrus, although needing to be refined, appears promising. Excessive non phloem material is eliminated, the exudate samples can be concentrated further, and consequently, the level of reaction improved. The relationship between the bacterial proteins of the Group 1 and Group 2 isolates and the proteins that occur in greening and dieback affected citrus can then be established.

Isolates obtained by Mochaba (1988) from South African greening, Reunion greening and Taiwan likubin affected plants, those isolated from a range of plants in the in planta collection at Beltsville (H.M. Garnett, personal communication) and the isolates from plants affected with ACD, fall into two distinct groups.

Group 1 consists of isolates from several different countries that share many properties. This group contains the SA01, SA03, SA05, SA06, RE01, TA01, TA02, AU01 and US04 isolates (as identified in the current study) and the NC II, GC I, GRL I, and K isolates (these were not available in the current study).

Group 2, consists of isolates obtained from South Africa, Australia and Beltsville, U.S.A., and includes isolates SA07, SA09, SA10, SA11, AU03, US02, US03, US05, US06, US07 and US08 (as identified in the current study) and LC I (not available in the current study).

The above distinction was first made by Mochaba (1988) based on isolation temperatures and subsequent optimal temperatures for growth in culture. This was

subsequently confirmed by the metabolic and biochemical tests. In the current study, the distinction of the above groups was further supported by morphological observations of broth and colony cultures, metabolic characterization, SDS-PAGE protein patterns, slot-blot immunoassay observations with antisera raised to isolates SA01, SA03, SA07 and RE01, western blot observations with the same antisera and a monospecific polyclonal antiserum raised to a common protein band observed in the Group 1 isolates.

Furthermore, the Group 2 isolates can be separated into two subgroups which include isolates SA07, AU03 and US02 (Subgroup 2a) and isolates SA09, SA10, SA11, US03, US05, US06, US07 and US08 (Subgroup 2b). It must be noted however, that not all the isolates in Subgroup 2b have been extensively studied and characterized and may consequently be regrouped as more information is accumulated.

The above groupings have furthermore been supported by an independent DNA homology and hybridization study comparing the same putative greening/dieback isolates (G. Hortelano Hap, personal communication).

Observations of the wall ultrastructure by D. Ariovich (Personal communication) suggested that all of the South African isolates studied in the current research could be the putative greening-associated organisms. This raises the question: which of the previously mentioned groups are actually associated with the greening disease of citrus?

However, to prove any association, Koch's postulates must be fulfilled. Hence it becomes necessary to introduce into citrus all the bacterial isolates obtained from greening affected citrus. Investigations in this respect were initiated by Mochaba (1988) who infected healthy seedlings with NC I, GC I, I, O and M (identified in the

current study as SA03, SA01 equivalent, RE01, TA01 and TA02, respectively), GRL I and LCI (not available in the current study). Each of these caused symptoms similar as those described for greening. Bacteria were subsequently isolated from plants infected with some of the isolates and found to be similar in many respects to the isolates originally injected. Infection of healthy seedlings with isolates SA01, RE01 and TA01 was also performed by H.M.Garnett (Personal communication) and greening-like symptoms occurred. Graft transmission of symptoms have also been successfully achieved. Hence, this group of organisms may be putative greening-associated agents.

Furthermore, it appears from comparison of the morphological, metabolic and serological observations of isolates AU01 and AU03 with the putative greening-associated isolates, that greening and ACD could be similar diseases. It is, therefore, important that while the AU01 and AU03 cultures are still available as low passage cultures, they be put back into citrus. Not only is the expression of symptoms required, but subsequent reisolation of bacteria identical to both AU01 and AU03 required to establish with certainty that these isolates are indeed associated with ACD.

The methods used in the current study appear to have satisfactorily distinguished between the putative greening/dieback-associated isolates and other Gram-negative bacteria and some plant pathogens. However, the scope of other bacteria tested in the current study were limited and many more comparative tests with a broader range of Gram-negative bacteria and plant pathogens need to be conducted.

The serological identification system developed in the current study appears to be reliable for distinguishing the putative greening/dieback-associated isolates in culture and also greening/dieback affected field samples in slot-blot immunoassays. Thus, identification of greening/dieback does not have to rely solely on symptoms,

which can be quite varied. Positive identification can be achieved by serological means. A point to note, however, is that the slot-blot immunoassay still has to be tested with a variety of other citrus diseases including co-infections by other agents, so that the diagnostic specificity of the antisera for greening/dieback can be determined.

It appears that by using the phloem extraction procedure, proteins occurring in the exudates can be extracted and detected by the IgG's. Refining this procedure and using more specific antisera could consequently yield information about the proteins that occur in the phloem tissue of greening/dieback affected plants. The protein patterns and subsequent serological reactions of these proteins can then be compared to those produced *in vitro* by the isolated bacteria. The association between the bacterial isolates available in culture and the agent *in situ* can furthermore be confirmed. In this way, the putative greening /dieback-associated isolates can also be distinguished from other agents that may occur in the diseased material, for example, citrus tristeza virus (Chen *et al.*, 1972).

Strains of citrus tristeza virus (CTV) have been identified by SDS-PAGE (Guerri *et al.*, 1990) and a monoclonal antibody has been developed that discriminates some CTV strains (Permar *et al.*, 1990). Affected citrus samples can then be investigated for the presence of either CTV proteins or greening/dieback proteins and the two agents further discriminated from one another. The sensitivity and specificity of this system could furthermore be improved by using a monoclonal antibody to the 38K - 40K protein band, the reaction of which can further be compared and contrasted to the reaction of the CTV monoclonal antibody.

Monoclonal antibodies are rapidly becoming useful tools in plant disease research (Halk and De Boer, 1985). Although the 38K - 40K protein was targeted in the current research, it was apparent from the western blot observations, in which the

Group 1 isolates were reacted with the anti-SA01, anti-SA03 and anti-RE01 IgG's, that there are at least five additional proteins in all the isolates that react equally with the three antisera. These proteins should consequently also be considered for monoclonal antibody research and the eventual development of a sensitive and highly specific detection system for greening/dieback affected citrus.

5.0 CONCLUSIONS

- A large group of bacterial isolates has been cultured from greening affected citrus and includes isolates from South African greening (SA01, SA03, SA05 SA06 and US04); Reunion greening (RE01); and Taiwan likubin (TA01 and TA02). Characteristics of these isolates, determined in the current study, suggest they are the same organism.

- The classification of these putative greening/dieback-associated isolates remains questionable and consequently will require further research.

- Bacterial isolates were obtained from ACD-affected citrus that resembled the Group 1 isolates.

- The reaction of preparations of greening affected citrus samples in slot-blot immunoassays with antisera raised to Group 1 isolates suggest that these isolates may indeed be the causal agents of greening.

- The detection of ACD affected citrus samples in slot-blot immunoassays with antisera raised against representative Group 1 isolates suggests greening and ACD to be similar diseases.

- Other isolates differing from the Group 1 isolates have also been obtained from greening affected citrus and placed in a separate group. These isolates do not all appear to be related and consequently sub groupings have been suggested. Not all the criteria investigated in the Group 1 isolates, in the current study, have been applied to the Group 2 isolates. Consequently, further studies still have to be conducted on the Group 2 isolates so that their association with greening/dieback, if any, can be established.

- The serological assays used in the current research linked the Group 1 isolates and have also successfully reacted with both greening and ACD affected field samples. The specificity and sensitivity of the serological techniques used in this study, however, still require refining so that the specific bacterial proteins observed in vitro can be detected in the phloem of affected tissues.

- While tests have been initiated in which some of the Group 1 isolates have been put back into plants and observed to develop symtoms, pathogenicity tests remain to be conducted on all the available isolates obtained from both greening and ACD affected citrus. Indeed, the association between the Group 1 isolates and Group 2 isolates, if any, to one another, and to affected greening/dieback citrus, remains to be established. Is it possible, for example, that the one group potentiates the symptoms induced by the other and consequently both groups found associated with the disease?

- The occurrence of other endophytic bacteria in citrus needs to be investigated.

APPENDIX I

Greening cultures : Original sources and previous identities

Culture	Source	Previous identity
SA01	– Leaves - Randburg (Mochaba, 1988) ...	G L
SA02	– Fruit - Randburg (Mochaba, 1988) ...	G C
SA03	– Fruit - Nelspruit (Mochaba, 1988) ...	NC I
SA05	– Fruit - Nelspruit (Mochaba, 1988) ...	NC III
SA06	– Isolated from seedlings infected with South African greening (Nelspruit source material), Beltsville collection (Mochaba, 1988) ...	E
SA07	– Fruit - Letaba (Duncan, 1985) ...	LC I
SA08	– Fruit - Letaba re-isolation (Mochaba, 1988) ...	LC 2
SA09	– Leaves - Tzaneen (Mochaba, 1988) ...	TZL
SA10	– Leaves - Letsitile (Mochaba, 1988) ...	LLL
SA11	– Leaves - Letsitile (Mochaba, 1988) ...	LL I
SA12	– Leaves - Brits (Mochaba, 1988) ...	BL

Note: The SA07 culture used in the current research is identified as LC - a highly subcultured isolate originally identified as LC I but which appear to have lost many of the characteristics of the original isolate.

RE01	– Isolated from seedlings infected with Reunion greening, Beltsville collection (Mochaba, 1988) ...	I
TA01	– Isolated from seedlings infected with likubin, Beltsville collection (Mochaba, 1988) ...	M

TA02 – Isolated from seedlings infected with likubin, Beltsville collection (Mochaba, 1988) ... 0

AU01 – Leaves - Gol Gol (Author's isolations) ... A_{31}

AU02 – Leaves - Nangiloc (Author's isolations) ... NJL

AU03 – Leaves - Dareton (Author's isolations) ... DL1

US01 – Isolate from plant injected with SA01 (H.M. Garnett, personal communication) ... No.26

US02 – Isolate from plant injected with TA02 (H.M. Garnett, personal communication) ... No.31

US03 – Isolate from plant injected with TA02 (H.M. Garnett, personal communication) ... No.35

US04 – Isolate from plant grafted with material derived from seedlings injected with SA01 (H.M. Garnett, personal communication) ... No.78

US05 – Isolate from plant with citrus yellow shoot (H.M. Garnett, personal communication) ... No.97

US06 – Isolate from plant grafted with material derived from seedlings infected with RE01 (H.M. Garnett, personal communication) ... No.158

US07 – Isolate from plant grafted with material derived from seedlings infected with SA01 (H.M. Garnett, personal communication) ... No.159

US08 – Isolated from plant grafted with material derived from seedlings infected with SA01 (H.M. Garnett, personal communication) ... No.164

APPENDIX II

Medium for the isolation of the putative greening-associated bacteria (MIG)

Constituents

Amino acids	(g/l)	Inorganic salts	(g/l)
L - alanine	0.500	KH_2PO_4	0.200
L - arginine	0.250	Na_2HPO_4	0.250
L - asparagine	0.150	$MgSO_4.7H_2O$	0.050
L - aspartic acid	0.350	NaCl	0.675
L - cysteine	0.400	$NaHCO_3$	0.020
L - glutamic acid	0.450	$CaCl_2.2H_2O$	0.450
L - histidine	0.300	$MgCl_2.2H_2O$	0.125
L - leucine	0.350	NH_4Cl	0.030
L - lysine	0.500	$CuSO_4.5H_2O$	0.005
L - methionine	0.350	$MnSO_4.4H_2O$	0.025
L - phenylalanine	0.250		
L - proline	0.200		
L - tryptophan	0.400		
L - valine	0.350		

(L - tyrosine 0.500)

Organic acids	**(g/l)**	**Carbohydrates**	**(g/l)**
α-ketoglutaric acid	0.800	Sucrose	16.50
Fumaric acid	0.650	Sorbitol	23.50
Malic acid	0.850		
Succinic acid	0.750		

Other	**(g/l)**
Tryptone	4.00
Brain heart infusion	3.00
Peptone	6.00
Yeast extract	5.00
Agar	15.0 g/l
Foetal calf serum	15% (v/v)

Companies from which the chemicals were obtained:

Amino acids and organic acids - SIGMA Chemical Company, St. Louis, MO, U.S.A.

Inorganic salts and carbohydrates - BDH Chemicals Pty Ltd, Kilsyth, Victoria, Australia.

Other and agar - OXOID Ltd, Basingstoke, Hanks., U.K.

Foetal calf serum - FLOW Laboratories, North Ryde, N.S.W., Australia.

Preparation

Complete and incomplete MIG broth

Mix the carbohydrates and other components in 400ml dH_2O and adjust the pH to 7.2 with 1.0M KOH. Sterilize by autoclaving at 121°C for 20 minutes and allow to cool. Dissolve the amino acids (except tyrosine) and inorganic salts in 325ml dH_2O by adding concentrated HCl. Similarly, the organic acids and tyrosine are dissolved in 75ml dH_2O by adding KOH pellets. The two mixtures are then slowly added to one another with stirring. After adjusting the pH to 6.2 with KOH, filter sterilize the solution using a pre-filter, 0.80µm, 0.45µm and 0.22µm millipore (Millipore Corporation, Bedford, M.A., U.S.A.) filter series. The carbohydrates and 150ml sterile foetal calf serum are then added and the medium stored at 4°C until use.

The procedure for making up incomplete MIG broth is the same as that described for the complete except that the foetal calf serum is omitted and replaced with 150ml sterile dH_2O pH7.5.

Sterility of the broths was confirmed by dispensing a small volume into a sterile flask which was incubated overnight at 25°C with shaking 150rpm.

MIG plates

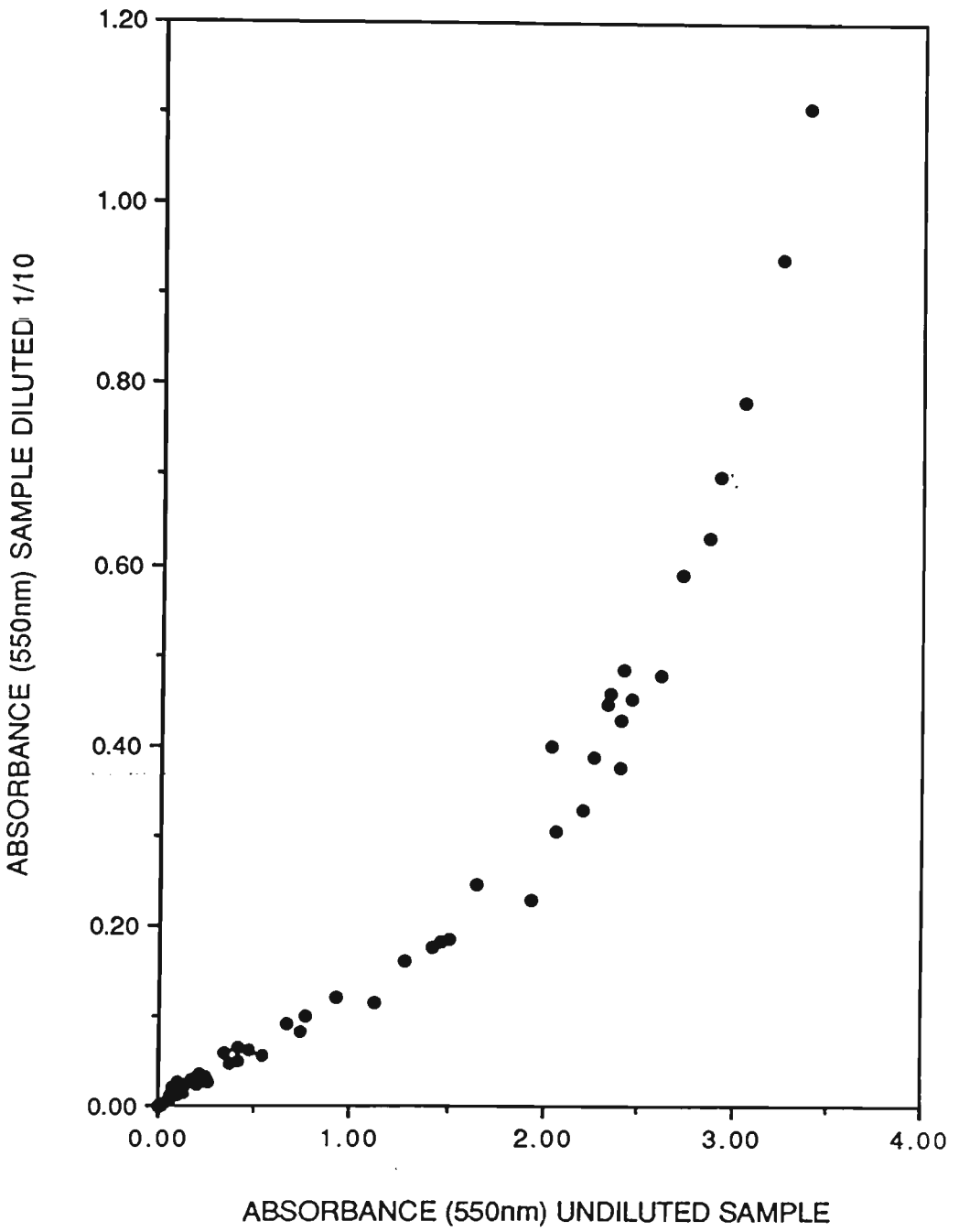
Agar, 15g, is added to the carbohydrates and the other components dissolved in 390ml dH_2O and the pH adjusted to 7.2 with KOH. After sterilizing at 121°C for 20 minutes, the solution is allowed to cool to ±60°C and mixed with the remaining components prepared as described for complete MIG broth. Either 150ml sterile foetal calf serum or 150ml sterile dH_2O is then added and the medium thoroughly mixed before pouring.

	MEAN	RANGE
CARBOHYDRATES AND OTHER COMPONENTS		
pH in 400ml dH_2O	6.91	6.79 - 7.01
Volume 1.0M KOH to adjust pH to 7.2	2.18ml	1.40 - 3.20ml
Final volume	437ml	432 - 445ml
AMINO ACIDS AND INORGANIC SALTS		
Volume conc HCl required to dissolve in 325ml dH_2O	5ml	----------
pH of solution	1.43	0.98 - 1.69
Final volume	332ml	330 - 335ml
ORGANIC ACIDS AND TYROSINE		
Mass KOH pellets to dissolve in 75ml dH_2O	4.34g	4.19 - 4.49g
pH of solution	13.18	13.02 - 13.45
Final volume	78.5ml	78.0 - 79.0ml
SOLUTION MIXTURE		
pH of mixture	3.52	2.76 - 3.77
Mass of KOH pellets and	2.33g	2.03 - 2.99g
volume 1.0M KOH to adjust pH to 6.2	1.26ml	0.9 - 2.15ml
Final volume	415ml	410 - 420ml
Volume foetal calf serum or dH_2O pH 7.5	150ml	----------
Final volume MIG medium	1001ml	998 - 1010ml
Final pH MIG medium	6.78	6.70 - 6.83

TABLE 3 - Quantitation of weights and volumes required for the preparation of MIG medium.

APPENDIX III

Reference standard curve for absorbance conversions



The standard curve relates the absorbance readings at 550nm (1cm light path) of undiluted growth curve samples to the absorbance of the same samples diluted 1/10 and read at the same wavelength (1cm light path) on a Bausch and Lomb 1001 spectrophotometer (Bausch & Lomb Inc, Rochester, N.Y., U.S.A.)

APPENDIX IV

Phosphate bufferd saline (PBS), 0.15M, pH 7.2

Dissolve 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 and KH_2PO_4 in 1 liter of dH_2O.

APPENDIX V

SDS Polyacrylamide gel electrophoresis

Stock solutions

Only electrophoretic grade (99%+ pure) chemicals were used

Acrylamide : bis-acrylamide 30% : 0.8%

Dissolve 29.2g of acrlylamide and 0.8g bis-acrylamide in, and make up to 100ml dH_2O, filter through Whatman No.1 filter paper. Store at 4°C.

10% Ammonium persulphate (APS)

Dissolve 0.1g in 1ml dH_2O. Make up fresh.

N,N,N'N'Tetramethylethylenediamine (TEMED)

Use undiluted.

Separating gel buffer (1.5M Tris HCl, pH8.8, 0.4% SDS)

Dissolve 18.17g Trizma base and 0.4g SDS in 75ml dH_2O. Adjust the pH to 8.8 using concentrated HCl and add dH_2O to a final volume of 100ml. Store at 4°C.

Stacking gel (0.5M Tris HCl, pH6.8, 0.4% SDS)

Dissolve 6.06g Trizma base and 0.4g SDS in 75ml dH_2O. Adjust the pH to 6.8 using concentrated HCl and add dH_2O to a final volume of 100ml. Store at 4°C.

Running buffer

To make up the 5x stock solution dissolve 15g Trizma base, 72g glycine and 5g SDS in and make up to 1litre dH_2O. For use dilute with dH_2O. Store at 4°C.

Sample buffer

dH_2O	4.0ml
0.5M Tris-HCl, pH6.8	1.0ml
Glycerol	0.8ml
10% SDS	1.6ml
2ß-mercaptoethanol	0.4ml
0.05% bromophenol blue	0.2ml

Dilute sample 1 : 4 with the sample buffer and heat to 100^{0}C by placing in a boiling water bath for 5 minutes.

Gel preparation (10.0% - 17.5% gradient system)

A 0.75mm thick 110 x 150mm separating gel was prepared as follows:

	10.0%	17.5%
Acrylamide stock	2.00ml	3.90ml
1.5M tris, pH8.8, 0.4% SDS	1.40ml	1.40ml
5% Glycerol	0.12ml	1.40ml
dH_2O	2.30ml	-----
	5.82ml	6.70ml

Degas for 15 minutes, add 31.3µl 10% APS and 3.13µl TEMED to each mixture and transfer to a gradient mixer and pour. Overlay the gel with approximately 1ml amyl alcohol and allow to polymerize at room temperature for 30 minutes. After polymerization rinse the top of the gel thoroughly with dH_2O and blot dry before adding the stacking gel. For a 1.5mm thick gel, double the volumes were used.

Stacking gel

Acrylamide stock	0.65ml
0.5M tris, pH6.8, 0.4% SDS	1.30ml
dH_2O	3.05ml
10% Ammonium persulphate	25µl
TEMED	5µl
	5.00ml

Pour the stacking gel preparation onto the separating gel, insert a 15 or 20 well comb and allow to polymerize for 30 minutes. Once the stacking gel has polymerized, remove the comb and rinse the wells with dH_2O. For a 1.5mm thick gel, double the volumes were used. Pre-electrophorese the gel in running buffer for 30 minutes before loading samples.

Running conditions

All the gels were run at room temperature, 125 V constant voltage for 5 hours (dye approximately 3 - 5mm from the bottom of the gel) to 6.5 hours (protein front approximately 10 - 15mm from the bottom of the gel).

APPENDIX VI

Slot-blot immunoassay and western blot buffers

TBS (Tris buffered saline - 20mM tris, 500mM NaCl and 0.02% Sodium azide, pH7.5.

Dissolve 2.42g tris, 26.24g NaCl and 0.2g NaN_3 in approximately 950ml of dH_2O. Adjust the pH to 7.5 with HCl and make up to 1litre with dH_2O.

TTBS (Tween-Tris bufferd saline)

Add 0.5ml Tween 20 to 1litre TBS

Blocking solution (3%)

Dissolve 3g gelatin (Purified grade) in 100ml TBS by warming to 37°C. Use at room temperature.

Antibody buffer (1%)

Dissolve 1g gelatin (Purified grade) in 100ml TBS by warming to 37°C. Use at room temperature.

Alkaline phosphatase reaction buffer (100mM tris, 100mM NaCl and 50mM $MgCl_2$, pH 9.5)

Dissolve 1.211g tris, 0.5844g NaCl and 1.0165g $MgCl_2$ in approximately 90ml dH_2O, adjust the pH to 9.5 with HCl and make up to 100ml with dH_2O.

APPENDIX VII

Biolog carbon utilization patterns

Table 4 lists the 95 carbon sources used in the Biolog GN microplates. The utilization patterns for the putative greening/dieback-associated bacterial isolates are also presented.

Colour reactions resulting from the oxidation of the carbon sources indicated by the conversion of tetrazolium to a highly coloured formazan were graded according to the intensity of the resulting purple colour. Absorbance readings were taken using a microplate reader set at 570nm to confirm the allocated colour gradings with reference to the A - 1 control well.

(+ + +) represents a strong purple colour, (+ +) for a weaker purple colouration and (+) where only a faint purple colour was discernable.

TABLE 4 – Biolog GN patterns

CARBON SOURCE	ISOLATES													
	SA01	SA03a	SA03b	SA05	SA06	SA11	RE01	TA01	TA02	AU01	AU02	AU04	AU05	US04
Water														
α-cyclodextrin														
Dextrin	+++	+++	+++	+++	+++		+++	+++	+++	+++	+	+++	+++	++
Glycogen													++	
Tween 40	+++	+	+++	+++	+++	+++	+++	+++	+++	+++		++	+++	++
Tween 80				++		++	+++		+	+++			++	
N-acetyl-D-galactosamine						++								
N-acetyl-D-glucosamine													++	
Adonitol						++								
L-arabinose	++	+++	+++	+++	++	+++	++	+	+	+	+++	+++	+++	+++
D-arabitol														
Cellobiose	+++	+++	+++	+++	++	++	+++	+++	+++	+		++	++	+++
I-erythritol														
D-fructose	+++	+++	+++	+++	+++		+++	+++	+++	+	+++	+++	+++	++
L-fructose														
D-galactose	+++	+++	+++	+++	++		+++	+++	+++		+++	+++	+++	+++
Gentiobiose	+++	+++	+++	++	+++		+++	+++	+++			++	+++	++
α-D-glucose	+++	+++	+++	+++	+++		+++	+++	+++	+++	+++	+++	+++	+++
M-inositol													++	
α-lactose													++	
Lactulose													++	
Maltose	+++	+++	+++	+++	+++		+++	+++	+++	+++	+++	+++	+++	+++
D-mannitol	+++	+++	+++				+++	+++	+++	+++	+++	+++	+++	++
D-mannose	+++	+++	+++	+++	+++		+++	+++	+++	+++	+++	+++	+++	+++

TABLE 4 – Continued

CARBON SOURCE	ISOLATES													
	SA01	SA03a	SA03b	SA05	SA06	SA11	RE01	TA01	TA02	AU01	AU02	AU04	AU05	US04
D-melibiose													+	
β-methyl glucoside													+	
Psicose	+++	+++	+++	+++	+++		++	++	++	+	+++	+++	+++	+
D-raffinose													+	
L-rhamnose														
D-sorbitol	++	++	+++		+		++	++	++		+++	+++	+++	++
Sucrose	+++	+++	+++	+++	+++		+++	+++	+++	+++	+		+	+++
D-trehalose	++	+++	+++	++	+++		+++	+++	+++					++
Turanose	+++	+++	+++	+++	+++		+++	+++	+++	+++		+++	+++	+++
Xylitol											+++	++	+++	
Methyl pyruvate	+++	+++	+++	+++	+++	++	+++	+++	+++	+++			++	+++
Mono-methyl succinate						++				++		++	+++	
Acetic acid		+	+	+	+	+++					+	+		
Cis-asconitic acid						++								
Citric acid														
Formic acid														
D-galactonic acid lactone														
D-galacturonic acid														
D-gluconic acid											+	+++	+++	+++
D-glucosaminic acid													++	
D-glucuronic acid														
α-hydroxy butyric acid					+		+							+
β-hydroxy butyric acid						+++								
γ-hydroxy butyric acid														

TABLE 4 – Continued

CARBON SOURCE	ISOLATES													
	SA01	SA03a	SA03b	SA05	SA06	SA11	RE01	TA01	TA02	AU01	AU02	AU04	AU05	US04
p-hydroxy phenylacetic acid	+++	++	++	+	+++		+++	+++	+	+++				+++
Itaconic acid														
α-keto butyric acid		+	++	+	+++	+++	++	+						+
α-keto glutaric acid														
α-keto valeric acid						+++								
D,L-lactic acid														
Malonic acid														
Proprionic acid		+	+		+	+++					+	++		++
Quinic acid														
D-saccharic acid														
Sebacic acid														
Succinic acid							+							
Bromo succinic acid		+	+	+	+		+							
Succinamic acid														
Glucuronamide														
Alaninamide						+++								
D-alanine						+++								
L-alanine						+++								
L-alanyl-glycine						+++								
L-asparagine		++				+++								
L-aspartic acid						+++								
L-glutamic acid						+++								
Glycyl-L-aspartic acid						+++								
Glycyl-L-glutamic acid						+++								

TABLE 4 – Continued

CARBON SOURCE	ISOLATES													
	SA01	SA03a	SA03b	SA05	SA06	SA11	RE01	TA01	TA02	AU01	AU02	AU04	AU05	US04
L-histidine						+++								
Hydroxy L-proline						+++								
L-leucine						+++								
L-ornithine						+++								
L-phenylalanine						++								
L-proline						+++								
L-pyroglutamic acid														
D-serine														
L-serine						+++								
L-threonine						+++								
D,L-camitine														
γ-amino butyric acid														
Urocanic acid														
Inosine														
Uridine														
Thymidine														
Phenylethylamine														
Putrescine	++	+	+				+	+	+					
2-amino ethanol														
2,3-butanediol														
Glycerol	+++	+++	+++	+++	+++		+++	+++	+++	++	+++	+++	+++	+++
D,L-α-glycerol phosphate														
Glucose-1-phosphate	+++	++	+	+			+++	++	++					
Glucose-6-phosphate		++	++	+			+++	++	++					

APPENDIX VIII

Growth curve statistical analyses

The exponential and stationary phase regions for both 25°C and 35°C for which the data were fitted and compared are listed in the following pages.

Analysis of variance (ANOVA) tables from which the comparative conclusions regarding any variations in the curves at the two temperatures were derived have also been included. The tables present the source of variation in question, the degrees of freedom (d.f.), the sum of squares (SS), the mean of squares (MS) and the F-statistic.

The p-value listed represents in each case, the probability of getting a value larger than the one observed by chance alone. The null hypotheses would be rejected only if $p \leq 0.05$.

ISOLATE SA01

Exponential phase data analysed included 6 replicate absorbance readings for each of the times T_{16}, T_{24} and T_{32} at 25°C and T_{8}, T_{16} and T_{24} at 35°C.
Stationary phase data analysed included 5 replicate absorbance values for each of the times T_{94}, T_{140}, T_{164}, T_{188} and T_{212} for both temperatures. Only 4 replicate and 2 replicate absorbance readings were entered for the 25°C temperature curve at times T_{188} and T_{212} respectively.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.33734	0.16867	
Different slopes rather than a common slope	1	0.00028	0.00028	0.18
Residual	32	0.05086	0.00159	
Total	35	0.38848		

p = 0.674

The F-value is significant at p = 0.674. The null hypothesis suggesting the exponential phases of growth at 25°C and 35°C have a common slope is accepted with a 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.01999	0.01999	12.74
Parallel (but not horizontal) lines	1	0.00000	0.00000	0.00
Lines which are not parallel	1	0.00010	0.00010	0.06
Residual	42	0.06590	0.00157	
Total	45	0.08598		

Non parallel lines	p = 0.808
Parallel (but not horizontal) lines	p = 1.000

As the F-value for non-parallel lines has a p-value of 0.808 and the F-value for parallel but non-horizontal lines has a p-value of 1.000, the hypothesis of horizontal line is accepted with a 5% level of significance. The p-value for testing the presence of two horizontal lines (as opposed to one) is 0.001 leading to the conclusion that there is a significant difference at the 0.1% level between the y-intercepts of the 25^{0}C and 35^{0}C lines.

ISOLATE SA03

The exponential phase data analysed included 2 replicate absorbance readings for each of the times T_{13}, T_{17} and T_{25} at 25°C. Single absorbance values were analysed at T_9, T_{13} and T_{17} for the 35°C exponential phase. The stationary phase data consisted of 2 replicate readings at 25°C and single readings at 35°C for the times T_{89}, T_{121} and T_{153}.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.06417	0.03208	
Different slopes rather than a common slope	1	0.00105	0.00105	5.84
Residual	5	0.00090	0.00018	
Total	8	0.06611		

p = 0.060

The F-value is significant at p = 0.060. The hypothesis of a common slope is accepted at the 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.04857	0.04857	271.25
Parallel (but not horizontal) lines	1	0.00062	0.00062	3.46
Lines which are not parallel	1	0.00175	0.00175	9.79
Residual	5	0.00090	0.00018	
Total	8	0.05184		

Non parallel lines p = 0.026

As the F-value for non parallel lines has a p-value of 0.026, the hypothesis that the two lines have equal slopes is rejected with a 5% level of significance suggesting that the lines have significantly different slopes. Each slope is also significantly different from the horizontal at the 5% level of significance.

ISOLATE SA05

The data analysed included triplicate absorbance values for each of the exponential phase times T_{16}, T_{24}, T_{40} and T_{48} and stationary phase times T_{96}, T_{144}, T_{192}, T_{240} and T_{288}. Data times and number of repetitions were the same for both temperatures.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.42045	0.21023	
Different slopes rather than a common slope	1	0.00153	0.00153	2.35
Residual	20	0.01302	0.00065	
Total	23	0.43500		

p = 0.141

The F-value is significant at p = 0.141. The hypothesis of a common slope is accepted with a 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.08673	0.08673	289.40
Parallel (but not horizontal) lines	1	0.00001	0.00001	0.04
Lines which are not parallel	1	0.00106	0.00106	3.53
Residual	26	0.00779	0.00030	
Total	29	0.09559		

Non parallel lines	p = 0.071
Parallel (but not horizontal) lines	p = 0.843

The F-value for non parallel lines has a p-value of 0.071 and the F-value for parallel but not horizontal lines has a p-value of 0.843. It can be concluded that the the two lines are horizontal at the 5% level of significance. The hypothesis of equal slopes is however, rejected at the 10% level. The p-value for two straight lines = 0.000 meaning that there is a significant difference between the two y-intercepts at the 0.1% level.

ISOLATE SA09

The data collected for the 25°C curve included 6 replicates for each of the corresponding times representing the exponential phase - T_8, T_{16}, T_{24}, T_{32} and T_{48} and the stationary phase -T_{80}, T_{96}, T_{128}, T_{152}, T_{211} and T_{253}. The 35°C data included 5 replicate absorbances for each of the exponential phase times T_8, T_{16}, T_{24} and T_{32} and stationary phase times T_{80}, T_{96}, T_{128}, T_{152}, T_{211} and T_{253}.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.54022	0.27011	
Different slopes rather than a common slope	1	0.01182	0.01182	6.24
Residual	46	0.08717	0.00190	
Total	49	0.63920		

p = 0016

The F-value is significant at p = 0.016. The hypothesis is therefore rejected with a 5% level of significance suggesting that the best fitting lines for the two slopes are significantly different.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.22232	0.22232	68.14
Parallel (but not horizontal) lines	1	0.05637	0.05637	17.28
Lines which are not parallel	1	0.03269	0.03269	10.02
Residual	62	0.20228	0.00326	
Total	65	0.51366		

Non parallel lines p =0.002

The F-value for non-parallel lines has a p-value of 0.002 suggesting that the lines are not parallel at the 0.5% level. The slopes of the lines can be concluded to be significantly different from one another. The slope for the line at 25°C is not significantly different from zero at the 5% level while the 35°C line is significantly different from zero at the 1% level.

ISOLATE SA10

The exponential phase data analysed included 6 replicate absorbance readings for each of the times T_{24}, T_{32}, T_{40} and T_{48} at 25°C and T_8, T_{16}, T_{24} and T_{32} at 35°C. The stationary phase data included 6 replicate obsorbance values for each of the times T_{96}, T_{112}, T_{164}, T_{188}, T_{212}, T_{260} and T_{284} analysed for both temperatures. T_{112} and T_{164} at 25°C had 5 replicate absorbance values.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.53455	0.26727	
Different slopes rather than a common slope	1	0.00040	0.00040	0.19
Residual	43	0.08912	0.00207	
Total	46	0.62406		

p = 0.665

As the F-value is significant at p = 0.665, the null hypothesis is accepted. The slopes of the exponential phases of growth at the two temperatures are equal at the 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.11494	0.11494	14.62
Parallel (but not horizontal) lines	1	0.06393	0.06393	8.13
Lines which are not parallel	1	0.00237	0.00237	0.30
Residual	78	0.61314	0.00786	
Total	81	0.79438		

Non parallel lines	p = 0.585
Parallel (but not horizontal) lines	p = 0.006

The F-value for non-parallel lines has a p-value of 0.585 and the F-value for parallel but non horizontal lines lines has a p-value of 0.006. The two lines can accordingly be concluded to be parallel with a common slope that is significantly different from 0. The p-value for testing the difference in the y-intercepts for the two lines is 0.0003 and significant at the 0.1% level.

ISOLATE TA01

The data analysed include triplicate absorbance values for each of the exponential phase times T_8, T_{16} and T_{24} and stationary phase times T_{64}, T_{80}, T_{104}, T_{128}, T_{152}, T_{176}, T_{200}, T_{224} and T_{248}.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.16100	0.08050	
Different slopes rather than a common slope	1	0.00000	0.00000	0.01
Residual	14	0.00777	0.00056	
Total	17	0.16877		

p = 0.922

The F-value is significant at p = 0.922. The hypothesis of a common slope is accepted at the 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.26938	0.26938	133.05
Parallel (but not horizontal) lines	1	0.04412	0.04412	21.79
Lines which are not parallel	1	0.01164	0.01164	5.75
Residual	50	0.10124	0.00203	
Total	53	0.42638		

Non parallel lines p = 0.020

The F-value for non-parallel lines has a p-value of 0.020 from which it can be concluded that the hypothesis for parallel lines is rejected with a 2.5% level of significance. The slopes for the two lines are significantly different. The slope of the line at 25°C is not significantly different from 0 at the 5% level while the slope of the line at 35°C is significantly different from 0 at the same level of significance.

ISOLATE TA02

The data analysed include triplicate absorbance values for each of the log phase times T_8, T_{16} and T_{24} and stationary phase times T_{80}, T_{96}, T_{136}, T_{184}, T_{232}, and T_{280} at both temperatures.

Analysis of variance: Exponential phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two parallel lines	2	0.16196	0.08098	
Different slopes rather than a common slope	1	0.00099	0.00099	2.11
Residual	14	0.00657	0.00047	
Total	17	0.16952		

p = 0.168

The F-value is significant at p = 0.168. The hypothesis of a common slope is accepted at the 5% level of significance.

Analysis of variance: Stationary phase

SOURCE OF VARIATION	d.f.	SS	MS	F
Two Horizontal lines	1	0.10368	0.10368	165.39
Parallel (but not horizontal) lines	1	0.00408	0.00408	6.50
Lines which are not parallel	1	0.02799	0.02799	44.65
Residual	32	0.02006	0.00063	
Total	35	0.15581		

Non parallel lines p = 0.000

The F-value for parallel lines has a p-value of 0.000 from which it can be concluded that the slopes of the two lines are significantly different at the 0.1% level of significance. Both lines have slopes that differ significantly from 0 at a 0.1% level of significance.

APPENDIX IX

Molecular weight determinations

Two factors had to be taken into consideration when the apparent molecular weights of the western blot protein bands were calculated.

1. The amido black staining procedure of the molecular weight markers and the blotting procedure applied to the remaining blot resulted in the nitocellulose being of differing lengths.

The difference in length of the nitrocellulose strip containing the markers and the remaining blot was measured giving a conversion factor with which to adjust the distances migrated by the markers measured on the original blot.

2. Conditions in the gel result in the standard curve not holding a straight line relationship when the log of the molecular weights are plotted against the distance migrated (Figure 58).

The handling of the data for each western blot consequently incorporates the use of a standard curve for the molecular weight range 94.0K - 43.0K (Figure 59) and the 43.0K - 14.4K range (Figure 60). The molecular weights of the unknown protein bands were then calculated from the straight line equation. The apparent molecular weights of protein bands within a range extending 5mm to either side of the distance migrated by the 43K molecular weight marker, were calculated by averaging the value calculated from both the 94.0K - 43.0K and 43.0K - 14.4K standard curves.

Standard curves were accordingly established for each blot.

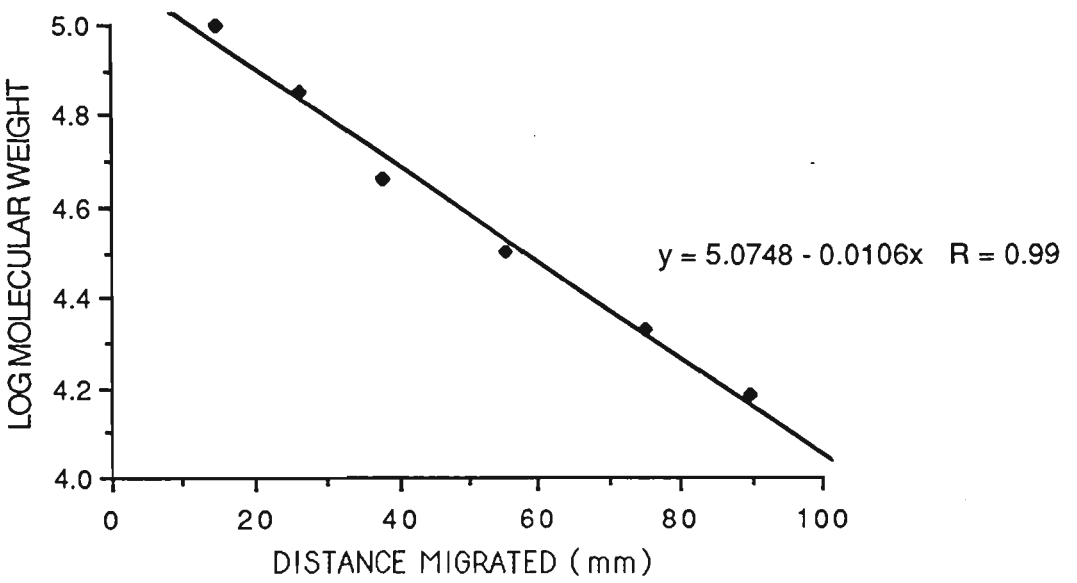


FIGURE 58 - Standard curve for the 94.0K - 14.4K low molecular weight markers

The logarithm of the molecular weights is plotted against the distance migrated by each band midpoint. The apparent molecular weights of unknown protein bands are calculated from the straight line equation.

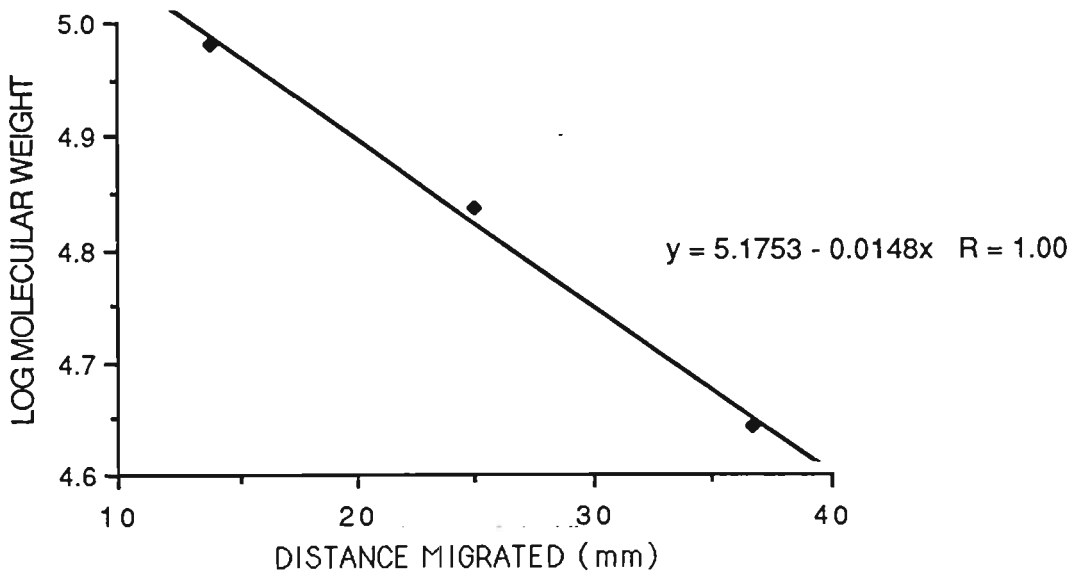


FIGURE 59 - Standard curve for the 94.0K - 43.0K low molecular weight markers

The logarithm of the molecular weights is plotted against the distance migrated by each band midpoint. The apparent molecular weights of unknown protein bands are calculated from the straight line equation.

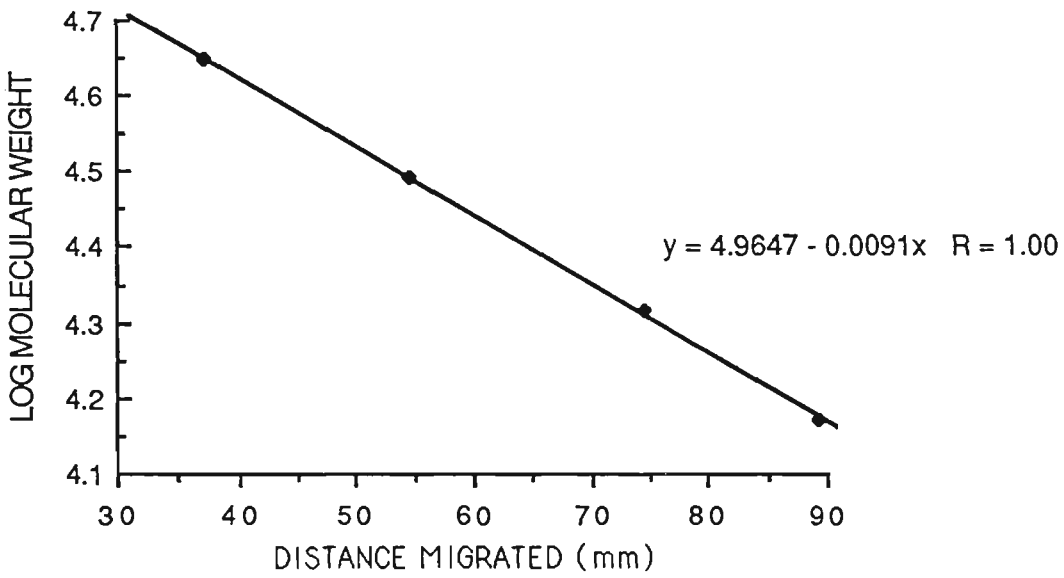


FIGURE 60 - Standard curve for the 43.0K - 14.4K low molecular weight markers

The logarithm of the molecular weights is plotted against the distance migrated by each band midpoint. The apparent molecular weights of unknown protein bands are calculated from the straight line equation.

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